Characterization of recombinant Modified Vaccinia virus Ankara expressing the Middle East respiratory syndrome coronavirus nucleocapsid protein

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ABBREVIATIONS

(h)DPP4	(Human) dipeptidyl peptidase 4
aa	Amino acid
ACE2	Angiotensin-converting enzyme 2
APN	Aminopeptidase N
ARDS	Acute respiratory distress syndrome
CEACAM1a	Carcinoembryonic antigen cell adhesion molecule 1a
CEF	Chicken embryo fibroblasts
CEPI	Coalition for Epidemic Preparedness Innovations
CVA	Chorioallantois Vaccinia virus Ankara
DNA	Deoxyribonucleic acid
E	Envelope protein
EFC	Entry fusion complex
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
ERGIC	Endoplasmic reticulum-Golgi intermediate compartment
EV	Extracellular virion
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
GFP	Green-fluorescent protein
HA	Hemagglutinin
HCMV	Human cytomegalovirus
HCoV	Human coronavirus
HIV	Human immunodeficiency virus
hpi	Hours post infection

i.m.	Intramuscular
i.p.	Intraperitoneal
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interleukin
ITR	Inverted terminal repetitions
kbp	Kilobase pairs
kDa	Kilo Dalton
M	Membrane protein
M1	Matrix 1 protein
MEM	Minimum Essential Medium Eagle
MERS-CoV	Middle East respiratory syndrome coronavirus
MHC	Major histocompatibility complex
MHV	Mouse hepatitis virus
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MV	Mature virion
MVA	Modified Vaccinia virus Ankara
N	Nucleocapsid protein
NA	Neuraminidase
NHP	Non-human primate
NP	Nucleoprotein
NYVAC	New York attenuated vaccinia virus
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEDV	Porcine epidemic diarrhea virus
PFU	Plaque-forming-unit
PMA	Phorbol myristate acetate
PRR	Pattern recognition receptor
RBD	Receptor binding domain
RNA	Ribonucleic acid
S	Spike protein
SARS-CoV	Severe acute respiratory syndrome coronavirus
SDS	Sodium dodecyl sulfate
SFC	Spot forming cells
TGEV	Transmissible Gastroenteritis virus
TNF	Tumor necrosis factor
VACV	Vaccinia virus
WHO	World Health Organization

I. INTRODUCTION

Emerging respiratory coronaviruses such as the severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) have caused worldwide epidemics with high morbidity, mortality and economic losses. MERS-CoV was first described in 2012 as a novel infectious agent causing severe and often fatal respiratory disease in humans. Up to date a total of 2279 confirmed cases with a fatality rate of 35% have been reported. Most of the epidemics are located in the Arabian Peninsula. In that context, dromedary camels are suspected to be the most important animal reservoir leading to zoonotic infections in humans. So far there are no therapeutics or candidate vaccines licensed. Most efforts in vaccine research against MERS-CoV focused on the viral spike (S) protein, which mainly elicits neutralizing antibody response. In contrast, there is still relatively little known about the role of T cell responses directed against MERS-CoV. Recently published data suggest that both antibody as well as T cell-based cellular immunity are crucial for viral clearance and recovery from MERS-CoV infection. Therefore, the highly conserved nucleocapsid (N) protein might be a potential target immunogen to elicit MERS-CoV-specific antibodies as well as cellular immune responses.

Modified Vaccinia virus Ankara (MVA) is a highly attenuated and replication deficient strain of vaccinia virus that serves as one of the most advanced recombinant poxvirus vectors in preclinical research and human clinical trials for developing new vaccines against infectious disease and cancer.

The aim of this project was to evaluate the capacity of a recombinant MVA-MERS-N candidate vaccine to activate specific T cells *in vivo* in BALB/c mice comparing different immunization routes. For this we analyzed MERS-CoV-N-specific T cell epitopes by using two-dimensional matrix peptide pools. Indeed, the recombinant MVA vector vaccine induced MERS-CoV-N-specific CD8+ T cell response. Here, we identified a decamer peptide epitope within the MERS-CoV N protein activating significant levels of CD8+ T cells. The results from this study will allow to elucidate the role of MERS-N-specific T cells for MERS-vaccine induced protection and also to better understand MERS-CoV

pathogenesis in more detail.

II. LITERATURE REVIEW

1. MERS-CoV: a new emerging pathogen

1.1. Epidemiology

In June 2012, almost ten years after the sudden arise of severe acute respiratory syndrome coronavirus (SARS-CoV), another novel virus of the family *Coronaviridae* emerged causing severe respiratory disease and death in humans (GRAHAM et al., 2013; COLEMAN & FRIEMAN, 2014): Middle East respiratory syndrome coronavirus (MERS-CoV). This novel human coronavirus was initially identified and isolated from the sputum of a 60-year-old Saudi man succumbing to acute severe pneumonia and renal failure (ZAKI et al., 2012; AL-TAWFIQ & MEMISH, 2014). The clinical picture of this first MERS-patient evoked the SARS-CoV outbreak in 2003 (ZAKI et al., 2012). In September 2012, a second case of severe acute respiratory illness caused by this novel coronavirus was confirmed in the United Kingdom. Here, a 49-year old man was transferred to intensive care in London from a hospital in Qatar showing symptoms of severe respiratory illness (BERMINGHAM et al., 2012; AL-OMARI et al., 2018). The experience gained during the SARS epidemic facilitated the rapid development of diagnostic methods for the detection of this new emerging pathogen (DROSTEN et al., 2003; BERMINGHAM et al., 2012). Interestingly, retrospective analysis revealed that the first recognized cluster outbreak of MERS-CoV had already occurred in Jordan in April 2012. Thereby, later retesting of blood and respiratory samples confirmed MERS-CoV as the causative agent for severe respiratory illness in 13 patients housed in the public hospital in Jordan (HIJAWI et al., 2013; AL-TAWFIQ & AUWAERTER, 2019). In contrast to the SARS epidemic in 2002/2003, MERS-CoV still continues to circulate and to cause disease and infections in humans, mostly linked to countries of the Arabian Peninsula, i.e. Jordan, Saudi Arabia, Qatar, Oman and the United Arab Emirates. Sporadic cases of MERS-CoV have also occurred in Europe, Africa and North America due to travel-associated infections in the Middle East. Interestingly, there was a large hospital-associated epidemic in the

Republic of Korea in 2015, with 186 confirmed cases of MERS-CoV infections and 38 related deaths. The index case of the Korea outbreak was a traveler who developed fever after returning from a trip through different countries of the Arabian Peninsula. Starting from this first patient, 26 more individuals had been infected leading to consecutive nosocomial transmission throughout the Republic of Korea (KOREA CENTERS FOR DISEASE & PREVENTION, 2015; OH et al., 2018). At present, a total of 2279 laboratory-confirmed cases of infections with MERS-CoV, resulting in 806 deaths (35.5%), have been reported in about 27 countries according to the World Health Organization (WHO) (WORLD HEALTH ORGANIZATION (WHO), 2019b).

Mechanisms of human-to-human transmission are not yet completely understood. However, transmission of the virus most likely occurs via respiratory droplet infection and close contact (AL-OMARI et al., 2018). The spread of MERS-CoV through the human population is characterized by three main features, including sporadic community cases, family clusters and healthcare-associated cluster outbreaks. Sporadic community cases are presumably acquired from non-human exposure. Family clusters can be traced back to contact with an infected family index case (AL-TAWFIQ & MEMISH, 2016). Hospital-associated infections are caused by transmission from patients and healthcare workers. According to epidemiological data, healthcare facilities play a major role in human-to-human transmission of MERS-CoV leading to cluster outbreaks. Reasons for the efficient and rapid spread of MERS-CoV within healthcare facilities are among other things delayed recognition, inadequate hygienic measures or insufficient accommodation of infected patients (AL-TAWFIQ & AUWAERTER, 2019). Thus, healthcare workers are the main individuals at risk for MERS-CoV infections (ASSIRI et al., 2013b; MEMISH et al., 2013; AL-TAWFIQ & MEMISH, 2014). The exact origin of MERS-CoV is still unclear, but dromedary camels are considered to be an important animal reservoir causing sporadic primary zoonotic infections in humans (SONG et al., 2019). High levels of MERS-CoV-specific antibodies as well as viral genome could be detected in dromedaries throughout the Arabian Peninsula and in Africa (REUSKEN et al., 2013; HAAGMANS et al., 2014; MEYER et al., 2014; RAJ et al., 2014a; REUSKEN et al., 2014b; MEYER et al., 2016). Infection of juvenile and immunological naïve animals with MERS-CoV

results in a mild and transient disease of the upper respiratory tract (DURAI et al., 2015; MEYER et al., 2016). Furthermore, recent studies showed that infected dromedary camels younger than one year of age shed large amounts of the virus from the upper respiratory tract (ADNEY et al., 2014). In addition, viral ribonucleic acid (RNA) as well as antibodies to MERS-CoV could be detected in the milk of lactating camel mares (REUSKEN et al., 2014a). The exact mode of zoonotic transmission is still unclear, but human infections may be acquired from camels (MEMISH et al., 2014; HUI et al., 2018).

1.2. Taxonomy, molecular biology and life cycle

MERS-CoV, a member of the family *Coronaviridae*, is assigned to the genus *Betacoronavirus*, which is subdivided into four lineages (A, B, C, D). MERS-CoV belongs to the lineage C species together with two phylogenetically closely related bat coronaviruses, namely Bat-Coronavirus HKU4 and Bat-Coronavirus HKU5 (WOO et al., 2012; CHAN et al., 2015). SARS-CoV is a lineage B Betacoronavirus. In contrast to SARS- and MERS-CoV, four other coronaviruses are known to cause human infections: human coronavirus (HCoV-) 229E, HCoV-HKU1, HCoV-NL63 and HCoV-OC43. Infections with these viruses are mainly associated with mild and self-limiting respiratory illnesses (FOUCHIER et al., 2004; VAN DER HOEK et al., 2004; PYRC et al., 2007; WOO et al., 2009).

Coronaviruses are enveloped, positive-sense single-stranded RNA viruses with genomes of 25 to 32 kilobase pairs (kbp) causing various diseases in humans and a broad range of animals, including avian species (PERLMAN & NETLAND, 2009; GRAHAM et al., 2013). Coronaviruses have the potential to cause epidemics in livestock resulting in large economic losses, e.g. the porcine epidemic diarrhea virus (PEDV) in swine populations (LAU & CHAN, 2015). Similar to other coronaviruses, the 5’-proximal two-thirds of the MERS-CoV genome (about 30kbp in size) typically encodes the large replicase-transcriptase-complex containing 16 non-structural proteins within open reading frame (ORF) 1a and 1b. The essential structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N) are encoded by the remaining 3’ one-

third of the genome (VAN BOHEEMEN et al., 2012; GRAHAM et al., 2013; CHAFEKAR & FIELDING, 2018). In addition to the structural proteins, the region downstream of ORF1ab also produces strain specific accessory proteins (ORF3, ORF4a/b, ORF5, ORF8b) (GRAHAM et al., 2013). The number, genomic organization and the function of these accessory genes are unique to the different coronaviruses. In terms of MERS-CoV, the non-structural proteins 4a and 4b are involved in inhibition of type-I interferon (IFN) signaling pathways and double-stranded RNA sensors, which enables evasion from the innate immune system (SHOKRI et al., 2019). The coronavirus virion is characterized by large protruding spikes on the surface of the virions representing peplomers of trimeric S proteins (GRAHAM et al., 2013; CHAN et al., 2015). The single-stranded RNA genome is sourrounded by N proteins in form of a helical nucleocapsid. The viral envelope is composed of the E, M and the characteristic S proteins (GRAHAM & BARIC, 2010).

The membrane-anchored trimeric S protein mediates virus attachment and entry into host cells, thereby initiating infection (LU et al., 2013; QIAN et al., 2013; CHAN et al., 2015). For the MERS-CoV, the S protein consists of the N-terminal receptor-binding subunit (S1) and the C-terminal S2 subunit, which facilitates membrane fusion via conformational changes after cleavage of S1 and S2 (QIAN et al., 2013). In contrast to all other known coronaviruses, MERS-CoV uses the human cell surface amino dipeptidyl peptidase 4 (DPP4, also known as CD26) as a functional receptor for cell entry (table 1) (GRAHAM et al., 2013; RAJ et al., 2013). After identification of DPP4 as the specific MERS-CoV receptor, the receptor binding domain (RBD) within the S1 subunit has been further analyzed. Antibodies against this RBD were shown to efficiently neutralize MERS-CoV infection (DU et al., 2013; JIANG et al., 2013; MOU et al., 2013).

Table 1: **Coronavirus receptor usage** (GRAHAM et al., 2013)

Host	Virus	Receptor
Human	HCoV-229E	Aminopeptidase N (APN)
	HCoV-NL63	Angiotensin-converting enzyme 2 (ACE2)
	HCoV-OC43	Sialic acid moieties
	SARS-CoV	ACE2
	MERS-CoV	Dipeptidyl peptidase 4 (DPP4)
Pig	Transmissible Gastroenteritis virus (TGEV)	APN
Dog	Canine coronavirus	APN
Cat	Feline coronavirus	APN
Mouse	Mouse hepatitis virus (MHV)	Carcinoembryonic antigen cell adhesion molecule 1a (CEACAM 1a)

Following viral entry into the host cell, the viral RNA is released into the cytoplasm followed by translation of ORF1a and 1ab to produce two polypeptides. Subsequently, proteases encoded by ORF1a cleave these two polypeptides resulting in 16 non-structural proteins, which build up the large RNA replicase-transcriptase-complex (SONG et al., 2019). In the next step of MERS-CoV life cycle negative-sensed RNA copies are produced to serve as a template for the production of new viral genomic RNA and for the discontinuous transcription of subgenomic messenger RNA (mRNA). Following translation of all structural and non-structural proteins, virion assembly takes place within the lumen of the endoplasmic reticulum–Golgi intermediate compartment (ERGIC) starting with genomic RNA being encapsidated by N protein to form a helical nucleocapsid (ZUMLA et al., 2015). After budding of the nucleocapsid into

vesicles, which harbor S, M and E proteins, new infectious virus is released via exocytosis (ZUMLA et al., 2015; SONG et al., 2019).

1.3. Clinical features and pathogenesis

Clinical manifestations of MERS-CoV infections in humans range from asymptomatic or mild respiratory disease to acute respiratory distress syndrome (ARDS) and multiorgan failure (YIN & WUNDERINK, 2018). The time from infection to onset of disease varies between two to 14 days (SENGA et al., 2017). The main presenting symptoms are fever, cough, shortness of breath, sore throat and myalgia (ASSIRI et al., 2013a; ASSIRI et al., 2013b; CHAN et al., 2015). Gastrointestinal symptoms are the most frequent extrapulmonary symptoms, including vomiting, diarrhea, nausea and abdominal pain (ASSIRI et al., 2013a; CHAN et al., 2015). Hospitalization of MERS-CoV patients associated with severe illness and fatal outcome is often linked to comorbidities of these individuals, such as diabetes, immunosuppression and chronic renal disease (AL-TAWFIQ et al., 2014). As is the case for SARS-CoV infection, elderly people are at higher risk for severe disease with fatal outcome (ASSIRI et al., 2013a). Asymptomatic individuals are thought to play a major role in virus transmission (AL-TAWFIQ & AUWAERTER, 2019). This is supported by the fact that a high percentage of camel workers is positive for MERS-CoV-specific immune response without showing any symptoms of severe disease. These camel workers with mild or subclinical MERS infections are hypothesized to be the origin of primary severe MERS cases in the community (ALSHUKAIRI et al., 2018).

The pathogenesis of MERS-CoV infection is not yet fully understood. However, the distribution and expression level of specific MERS-CoV entry-receptor within the different human tissues and organs explains the clinical severity of MERS-CoV disease. The MERS-CoV entry-receptor DPP4 is a type II transmembrane glycoprotein and it is highly expressed on epithelial and endothelial cells of various tissues, including lung, liver, kidney, small intestine, and on activated leukocytes (LAMBEIR et al., 2003; CHAN et al., 2015; WIDAGDO et al., 2016). The significant higher expression of DPP4 in the kidney and alveoli explains the

clinical picture of the severe disease with renal dysfunction and highly lethal pneumonia. Interestingly, there is a different pattern of DPP4-expression in the upper respiratory tract between camels and humans. Widagdo and coworkers observed that the viral receptor is highly expressed on epithelial cells in the upper respiratory tract of camels giving the reason for efficient virus shedding and camel-to-camel transmission (WIDAGDO et al., 2016). Compared to dromedary camels, in humans DPP4-expression is limited to the lower respiratory tract. The lack of DPP4 in the human upper respiratory tract may explain the restricted and sporadic MERS-CoV transmission from human to human (WIDAGDO et al., 2016). This is also supported by the observation that large amounts of infectious virus could be detected in nasal secretions of MERS-CoV infected camels, but not in those of human MERS-patients (ADNEY et al., 2014; DROSTEN et al., 2014). Experimental MERS-CoV infected camels developed mild and transient respiratory disease, mainly limited to the upper respiratory tract, with nasal discharge, which persisted up to two weeks after challenge infection. High titers of infectious MERS-CoV could still be detected in nasal swabs obtained seven days after MERS-CoV challenge infection (ADNEY et al., 2014).

As described above, cells of the human respiratory tract are the primary infection site of MERS-CoV. This feature highlights the zoonotic potential of this new emerging pathogen (KINDLER et al., 2013). The infection of human respiratory epithelial cells results in robust viral replication (CHAN et al., 2013c; GRAHAM et al., 2013; KINDLER et al., 2013; ZHOU et al., 2015). Remarkably, the virus suppresses the induction of antiviral and proinflammatory cytokines. This observation could be confirmed in *ex vivo* lung tissues (LAU et al., 2013; ZIELECKI et al., 2013; ZHOU et al., 2015). Several MERS-CoV proteins, including the M protein and accessory proteins ORF4a/b and ORF5 have been identified as potent IFN antagonists (NIEMEYER et al., 2013; YANG et al., 2013; MATTHEWS et al., 2014; SIU et al., 2014). Unlike SARS-CoV, MERS-CoV is also able to infect and replicate in human macrophages, dendritic cells and T lymphocytes leading to disruption of the immune system with aberrant production of proinflammatory cytokines and T cell apoptosis (CHU et al., 2014; ZHOU et al., 2014; ZHOU et al., 2015; CHU et al., 2016). In addition to the lower respiratory tract epithelium, cells of the human intestinal tract were

recently shown to be susceptible for MERS-CoV suggesting that the human intestinal tract may serve as alternative infection route (ZHOU et al., 2017). The broad range of human tissue tropism and the ability to evade the host innate immune response associated with cytokine dysregulation may explain the possible clinical severity of MERS-CoV infections (CHAN et al., 2013b; CHAN et al., 2015).

1.4. Treatment and prevention

As there is currently no licensed vaccine or antiviral therapy available for MERS-CoV disease, supportive and palliative care remains the only mainstay of treatment for patients with severe MERS disease. Supportive care is mainly based on the provision of organ support and the management of complications including assisted ventilation, renal replacement therapy, fluids and antimicrobials to prevent secondary nosocomial and opportunistic infections especially in individuals with comorbidities (CHAN et al., 2015; CHAFEKAR & FIELDING, 2018).

Animal models are essential for the development and testing of safe and effective countermeasures against infectious diseases. Different animals have been already tested as models for human MERS disease progression and to evaluate the efficacy of antivirals and candidate vaccines (SUTTON & SUBBARAO, 2015; CHAFEKAR & FIELDING, 2018). In contrast to SARS-CoV, commonly used laboratory animals, including ferrets, the Syrian hamster or wild-type mice, are not suitable as MERS-CoV infection animal models due to their differences in the functional host receptor DPP4 (DE WIT et al., 2013a; COLEMAN et al., 2014; RAJ et al., 2014b; HAAGMANS et al., 2015; VAN DOREMALEN & MUNSTER, 2015). Rabbits are susceptible for MERS-CoV infection, since virus could be detected in the lungs and respiratory excretions of infected rabbits. However, there are no histopathological changes or symptoms of clinical disease observed in MERS-CoV infected rabbits (HAAGMANS et al., 2015). Also non-human primates (NHPs), such as rhesus macaques and common marmosets, are susceptible for MERS-CoV infection (DE WIT et al., 2013b; MUNSTER et al., 2013; FALZARANO et al., 2014; YAO

et al., 2014). However, the extent of viral replication and clinical outcome of disease varies in between both human primate species. Infection of rhesus macaques with MERS-CoV results in a mild clinical disease associated with transient pneumonia, whereas the infection of common marmosets causes lethal pneumonia simulating the more severe illness of human infections (SUTTON & SUBBARAO, 2015). The development of a suitable mouse model facilitates efficacy studies of candidate vaccines against MERS-CoV. Several adapted mouse models have been tested for the ability to simulate human MERS-CoV disease (GRAHAM et al., 2013; SUTTON & SUBBARAO, 2015). In the first model, an adenoviral vector expressing the DPP4 was introduced intranasally to BALB/c mice inducing a transient expression of the receptor in the lungs. Five days later mice had been intranasally challenged with a dose of $1x10^5$ plaque-forming-units (PFU) MERS-CoV. DPP4-transduced mice developed mild pulmonary disease starting about two to four days after MERS-CoV challenge infection (ZHAO et al., 2014; BASELER et al., 2016). Interestingly, clinical disease was more severe when using type-I IFN immunodeficient mice (ZHAO et al., 2014). In a second model, human DPP4-transgenic mice were generated to express the functional host cell receptor systemically. After challenge infection with MERS-CoV, DPP4-transgenic mice developed severe progressive respiratory disease with a fatality rate of 100% (AGRAWAL et al., 2015). However, high infectious virus titers could be detected in the brain of infected transgenic mice (AGRAWAL et al., 2015). In another transgenic mouse model approach, the mouse DPP4 ORF was replaced by the human DPP4 encoding sequence ensuring the physiological expression of human DPP4 (DPP4-humanized mice) (PASCAL et al., 2015; CHAFEKAR & FIELDING, 2018). But yet, as the mice remained asymptomatic after challenge infection with MERS-CoV Jordan strain, the DPP4-humanized mouse model could only be considered as a model to mimic mild human disease (PASCAL et al., 2015; BASELER et al., 2016). In 2019, Iwata-Yoshikawa and coworkers developed the most recent DPP4-transgenic mouse model for MERS-CoV infection. Here, expression of human DPP4 is under control of an endogenous promoter. As a result, the human MERS-CoV receptor is expressed in the lung and the kidney, displaying the expression pattern in humans. However, human DPP4-expression could also be detected in T cells within lymphoid tissues,

which is different to humans. After intranasal challenge infection with MERS-CoV, young and adult DPP4-transgenic mice only showed transient weight loss and they developed lower respiratory tract infection with acute multifocal interstitial pneumonia (IWATA-YOSHIKAWA et al., 2019). In a third approach, DPP4-chimeric mice were generated to be susceptible for MERS-CoV infection. Here, the mouse DPP4 encoding sequence was modified by two human amino acids. Infection of these DPP4-chimeric mice with a mouse-adapted MERS-CoV strain resulted in a ARDS-like respiratory disease characterized by extreme weight loss, decreased survival, decreased pulmonary function and pulmonary hemorrhage (COCKRELL et al., 2016). In summary, mice expressing the human functional host cell receptor DPP4 are susceptible to MERS-CoV infection and they serve as a suitable animal model for evaluating immunogenicity and efficacy of candidate vaccines, although each mouse model has its own benefits and limitations (BASELER et al., 2016).

Since the emergence of MERS-CoV, significant efforts have been made to develop effective antiviral agents. Based on the fact that MERS-CoV infection results in the production of antiviral cytokines in different cell lines, several studies investigated *in vitro* the antiviral effect of IFNs on viral replication (CHAN et al., 2015). Type-I IFNs, such as IFN-α, pegylated IFN-α and IFN-β were shown to reduce MERS-CoV replication in various cell lines demonstrated by a reduced cytopathic effect (CHAN et al., 2013a; DE WILDE et al., 2013; FALZARANO et al., 2013; KINDLER et al., 2013; ZIELECKI et al., 2013). The antiviral effect of IFNs on MERS-CoV infection was also investigated in animal studies. Inter alia, combined treatment of MERS-CoV-infected rhesus macaques with IFN-α2b and the RNA-polymerase inhibitor Ribavirin resulted in a reduced viral replication associated with moderate immune response and a mild clinical outcome. However, in this study the animals were treated within a few hours after MERS-CoV challenge infection. Therefore, combinational treatment of IFN-α2b and Ribavirin should be considered suitable for human patients in early stages of disease as it might happen in a hospital associated outbreak (FALZARANO et al., 2013; KHALID et al., 2015; CHAFEKAR & FIELDING, 2018). Another approach of antiviral treatment is to develop candidate drugs, which block viral attachment and viral entry into host cells. Therefore, the S protein and the corresponding viral attachment and cell entry

mechanisms are of particular interest as target antigens. Inhibition of virus attachment and entry into host cells can be achieved by monoclonal antibodies targeting the RBD. These monoclonal antibodies competitively block the binding of the RBD to the host cell receptor DPP4 with higher affinity than the RBD itself (DU et al., 2014; JIANG et al., 2014; YING et al., 2014; CHAN et al., 2015). The protective role of neutralizing antibodies could also be confirmed in different *in vivo* studies. Treatment or prophylactic intervention with neutralizing monoclonal antibodies resulted in a reduction of viral titers in the lungs of DPP4-transgenic mice, rabbits or non-human primates (AGRAWAL et al., 2016; HOUSER et al., 2016; JOHNSON et al., 2016; OKBA et al., 2017). In another study, DPP4-transduced mice were protected against MERS-CoV challenge infection due to passive immunotherapy with convalescent camel sera (ZHAO et al., 2015). In addition to antibodies inhibiting viral attachment and cell entry, specifically *in silico* designed antiviral peptides have been used to interact with the S2 subunit. These peptides prevented cell-to-cell fusion and viral entry into host cells in several *in vitro* studies (GAO et al., 2013; LU et al., 2014). The entry process of MERS-CoV into host cells is also inhibited by agents targeting the functional host cell receptor DPP4 (CHAN et al., 2015). Raj and coworkers demonstrated that adenosine deaminase, a DPP4 binding protein, acts as natural antagonist for MERS-CoV infection (RAJ et al., 2014b).

Nevertheless, the efficacy of antiviral candidate drugs remains uncertain in humans. For this, safe and efficient vaccines are urgently needed to prevent MERS-CoV infections in humans and animals (dromedaries) considering the insufficient intervention therapies for patients with severe MERS disease. Among all four structural proteins, the S and N protein are the major immunogenic target antigens of coronaviruses (AGNIHOTHRAM et al., 2014; OKBA et al., 2017). As described above, neutralizing antibodies are mainly directed against epitopes within the RBD region of the S protein (MOU et al., 2013). Thus, current candidate vaccines against MERS-CoV have been developed mainly focusing on the S protein. Therefore, different platforms were used to generate anti-S candidate vaccines, such as viral-vector-based vaccines, DNA vaccines, protein-based vaccines or whole virus vaccines (OKBA et al., 2017). DNA-based vaccines targeting the S protein were shown to evoke antigen-specific neutralizing antibodies as well as potent cellular immune

response in mice, NHPs and camels (MUTHUMANI et al., 2015; WANG et al., 2015a; WANG et al., 2016). Furthermore, a DNA-based vaccine proved to be protective in NHPs (MUTHUMANI et al., 2015; WANG et al., 2015a). For the generation of recombinant MERS-vaccines expressing the S protein (or parts of the S protein) several vector viruses have been used: Modified Vaccinia virus Ankara (MVA), adenovirus, measles virus, parainfluenza virus and rabies virus (KIM et al., 2014; MALCZYK et al., 2015; VOLZ et al., 2015b; HAAGMANS et al., 2016; WIRBLICH et al., 2017). Recombinant vector vaccines based on MVA, measles virus and rabies virus were shown to be protective against MERS-CoV challenge infection in mice, and in case of the MVA-based vaccine also in dromedary camels (MALCZYK et al., 2015; VOLZ et al., 2015b; HAAGMANS et al., 2016; WIRBLICH et al., 2017). Immunization with protein-based vaccines proved to be immunogenic in different animal species, including mice, rabbits and NHPs (OKBA et al., 2017). But even though protein-based vaccines have the highest safety profile, immunogenicity and efficacy largely depends on dose, immunization regime and the addition of a suitable adjuvant (WANG et al., 2016; OKBA et al., 2017).

Despite the promising data of current vaccine candidates inducing anti-MERS-CoV S immune responses, waning of virus-specific humoral immune responses and the development of antibody escape mutants should be considered critical for the generation of a long-lived anti-MERS vaccine (SUI et al., 2014; TANG et al., 2014). Furthermore, it is known that a combination of both virus-specific cellular and humoral immune responses is involved in protective immunity against coronaviruses in general (OKBA et al., 2017). Therefore, the highly conserved N protein is another promising target immunogen for vaccine development with the potential to induce a longer lasting cellular immune response.

2. Modified Vaccinia virus Ankara (MVA) as viral vector vaccine against MERS-CoV

2.1. History of MVA

MVA is a highly attenuated vaccinia virus (VACV) strain generated by continuous passages on primary chicken embryo fibroblasts (CEF) with the approach to develop a safer vaccine against human smallpox. VACV, the ancestor virus of MVA, has been used as vaccine against human smallpox for over 200 years. Unfortunately, severe side effects appeared when using VACV, which sometimes even lead to death (MAYR, 2003; GILBERT, 2013). The major side effects of VACV include dangerous localized reactions, such as eczema vaccinatum or generalized vaccinia, or even postvaccinal encephalitis, which can result in death (MAYR, 2003). MVA is derived from the VACV strain Ankara, which was used as smallpox vaccine in Turkey (MAYR & MUNZ, 1964; MAYR et al., 1975; VOLZ & SUTTER, 2017). In 1953, Mayr and Herrlich (from the Institute of Medical Microbiology, Infectious and Epidemic Diseases in Munich) started to propagate the parental virus of MVA on the chorioallantoic membranes of embryonated chicken eggs leading to the name Chorioallantois Vaccinia virus Ankara (CVA) (MAYR et al., 1975; VOLZ & SUTTER, 2017). Further amplification of CVA through over 500 serial passages on CEF cells resulted in a highly attenuated virus with reduced virulence and which was no longer able to replicate productively in human and most other mammalian cell lines (CARROLL & MOSS, 1997; DREXLER et al., 1998; MEISINGER-HENSCHEL et al., 2007). Compared to its ancestor virus CVA, MVA lost approximately 15% of the genome including six major deletions sites as well as a couple of small deletions and mutations affecting genes with functions in virus-host interactions (MEYER et al., 1991; ANTOINE et al., 1998; VOLZ & SUTTER, 2017). Preliminary clinical testing in humans conducted during the final stages of the smallpox vaccination campaign in South Germany confirmed the safety profile of MVA. Here, more than 120,000 individuals got vaccinated with MVA without documentation of severe side effects (STICKL et al., 1974; MAYR et al., 1978; MAHNEL & MAYR, 1994). Thereby, MVA vaccination proved to be safe for intracutaneous, subcutaneous and intramuscular

injections. Of note, MVA was even safe in immunocompromised individuals, in contrast to vaccination with replication competent VACV strain Elstree, which showed a drastic increase in virulence in those individuals (MAYR et al., 1978). To date, MVA is licensed in the European Union and Canada as a standalone third-generation smallpox vaccine for active immunization in adults, even for high-risk individuals (VOLLMAR et al., 2006; KENNEDY & GREENBERG, 2009; GREENBERG et al., 2013).

2.2. Taxonomy and viral life cycle

The family *Poxviridae* is divided into the two subfamilies *Chordopoxvirinae* (vertebrate specific) and *Entomopoxvirinae* (insect specific). The *Chordopoxvirinae* are subdivided into nine genera and two unassigned species, with *Orthopoxvirus* as the best-known genus. The most famous members within the genus *Orthopoxvirus* are variola virus, the causative agent of human smallpox disease, and VACV, as the prototypic member of the poxvirus family and the parental virus of MVA (MOSS et al., 2007). Poxviruses are large enveloped and barrel-shaped viruses containing linear double-stranded deoxyribonucleic acid (DNA) genomes that vary from 130 to 300 kbp. Due to their large dimension of about 250x360nm poxviruses can just be visualized by light-microscopy. The internal structure of the virions is characterized by a core containing the s-shaped genome, two lateral bodies and the outer lipid membrane (figure 1). The genome consists of a highly conserved central region encoding genes required for viral replication, and the end terminal regions containing ORFs associated with host interactions (MOSS, 1996; MOSS et al., 2007; WERDEN et al., 2008). The two double-stranded DNA strands are flanked by inverted terminal repetitions (ITR), which form two hairpin loops at the ends of the genome (BAROUDY et al., 1982).

Notably, two different forms of infectious particles are produced by poxvirus replication: intracellular mature virions (MVs), which represent the basic form of infectious particles with a single outer membrane, and extracellular enveloped virions (EVs), which are surrounded by an additional outer lipid membrane containing proteins distinct to MVs (SMITH et al., 2002; MOSS, 2006; MOSS et

al., 2007). The majority of infectious particles is represented by MVs. MVs are released via cell lysis and mediate infection between hosts, whereas EVs are required for cell-to-cell and long-range dissemination (BLASCO & MOSS, 1992; SMITH et al., 2002).

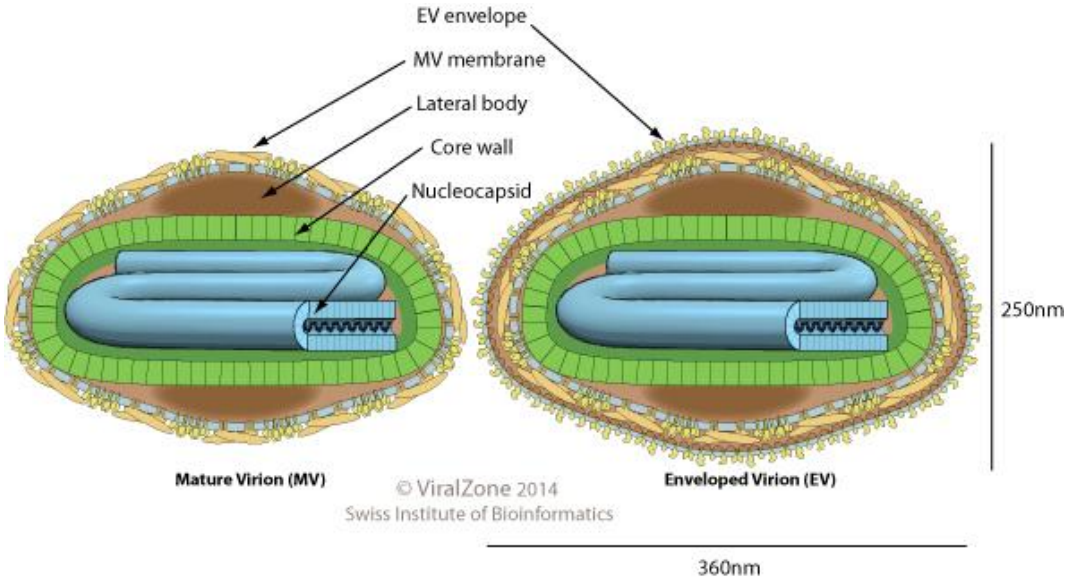

Figure 1: **Morphology of poxvirus virions**

(Source: ViralZone; www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics; with permission)

Interestingly, poxviruses replicate entirely within the cytoplasm of infected cells using their own transcription machinery (MOSS, 1996). The process of genome replication and virion assembly is typically regulated by a cascade mechanism depending on the level of expressed genes (figure 2) (MOSS, 1996; BROYLES, 2003). Initially, cell entry of MVs occurs via fusion with the plasma membrane or endocytosis, whereas EV-entry is mediated by disruption of the outer viral membrane leading to fusion of the inner viral membrane as described for MVs entry mechanism (LAW et al., 2006; MOSS, 2016). Compared to many other viruses, which use only one or two proteins for cell binding, fusion and entry, poxviruses encode a large number of proteins mediating virus entry process (MOSS, 2012). The attachment of VACV MVs is mediated by four proteins, which bind to glycosaminoglycans or laminin on the cell surface. Eleven more proteins, embedded in the MV membrane, the so called entry fusion complex (EFC), are involved in membrane fusion and core entry (MOSS, 2016). After cell entry, the virus core is released into the cytoplasm and early gene

expression starts immediately with early mRNA being detected within 20 minutes (MOSS et al., 2007). Proteins essential for the transcription of early genes are already packaged with the viral genome in the core, including a DNA-dependent RNA polymerase, early transcription factors, capping and methylating enzymes, and a poly(A)-polymerase (MOSS & EARL, 2001). Early genes encode proteins and transcription factors, which are essential for viral replication and intermediate gene transcription (MOSS, 1996; BROYLES, 2003). This leads to disruption of the viral core and the viral DNA is released into the cytoplasm, which is called uncoating (MOSS & EARL, 2001; MCFADDEN, 2005). In the next step, DNA replication takes place followed by successive transcription of intermediate as well as late genes within the so called viral factories (KATSAFANAS & MOSS, 2007). Intermediate as well as late class gene products are essential for virion morphogenesis and assembly including structural proteins, which are encoded by late genes (BROYLES, 2003). Late genes encode also for early transcription factors, which are then packaged with the viral genome (MOSS & EARL, 2001). Finally, the structural proteins and newly synthesized viral genome copies assembly to form MVs. In the next step, these MVs are further enwrapped by two membranes derived from the trans-Golgi network to form intracellular enveloped virions (HILLER & WEBER, 1985; SCHMELZ et al., 1994). These enveloped virions move to the cell membrane and are released as EVs via fusion with the plasma membrane (BLASCO & MOSS, 1992; CUDMORE et al., 1995; WARD & MOSS, 2001).

A special feature of MVA is that MVA is replication deficient in mammalian cells. Here, the life cycle is blocked during late gene expression at the stage of virion assembly. In detail, MVA virion assembling is inhibited at the stage of proteolytic processing of late viral proteins resulting in immature virus particles. However, the pattern of early and late proteins is similar to replicating VACV strains with late genes being expressed between six and 12 hours after cell infection (SUTTER & MOSS, 1992).

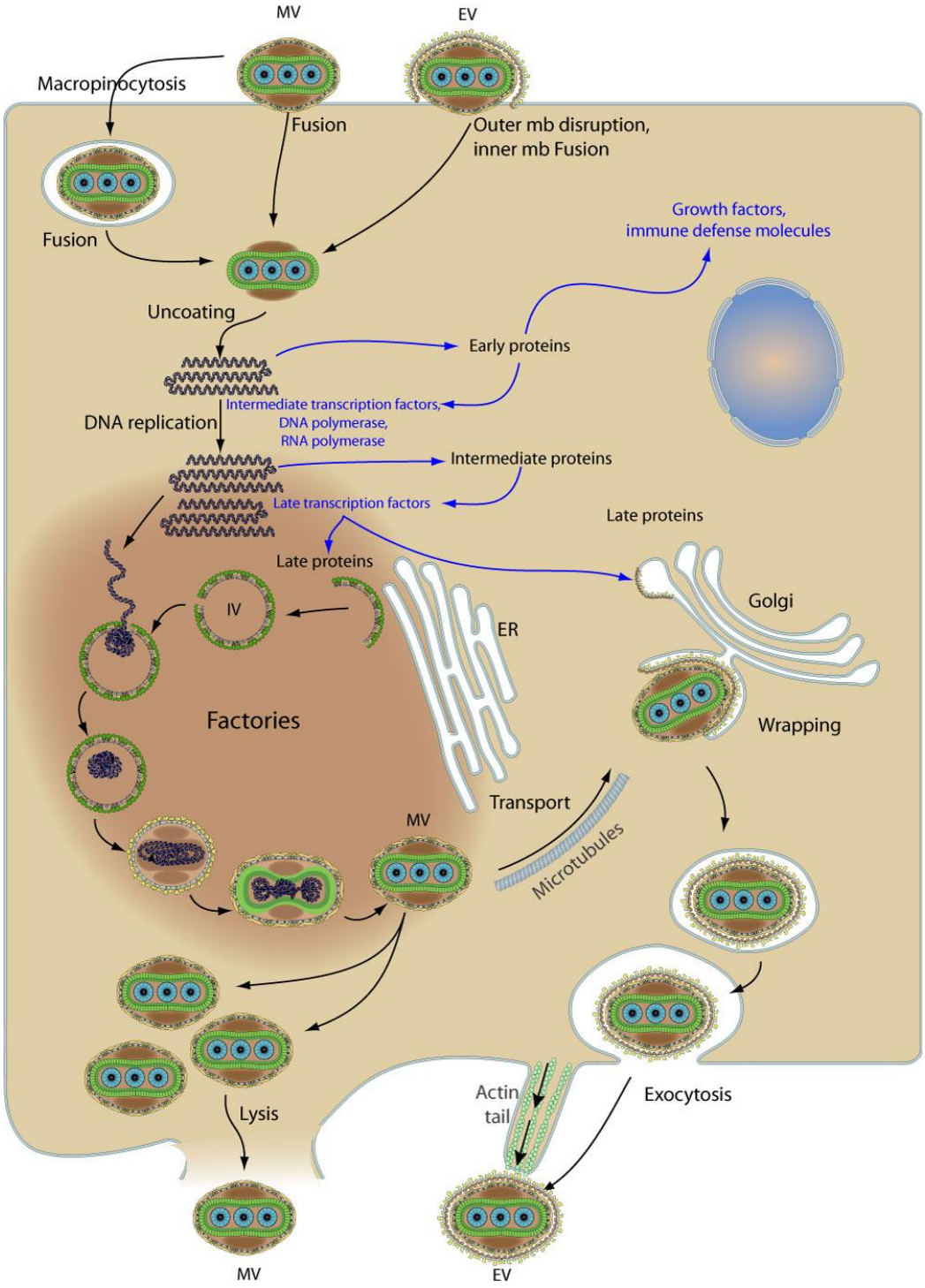

Figure 2: **Replication cycle of poxviruses**

(Source: ViralZone; www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics; with permission)

2.3. MVA as a viral expression vector

Smallpox vaccination discontinued after the eradication of human smallpox was officially declared in 1980 (FENNER, 1993). Since 1982, poxviruses and especially the prototype Orthopoxvirus VACV have been established as recombinant expression vectors in vaccine development (MACKETT et al., 1982; PANICALI & PAOLETTI, 1982; MOSS, 1996). In general, poxviruses exhibit several advantages, which make them suitable for the application as viral vector vaccine (SMITH & MOSS, 1983; PERKUS et al., 1985; MOSS, 1996; DRAPER et al., 2013; KREIJTZ et al., 2013; VOLZ et al., 2015a; VOLZ & SUTTER, 2017): i) Due to their own large genome and the high genetic plasticity, poxviruses are able to incorporate large (at least 25kbp) or even multiple foreign genes into their genome. ii) The replication cycle of poxviruses, unlike other DNA viruses, is limited to the cytoplasm of infected host cells. Thus, gene expression is under strict control of virus-specific transcription systems, without any integration of viral DNA into the host genome. iii) Regarding the immunogenicity and efficacy as vector vaccines, poxviral vectors are able to induce strong adaptive immunity, namely cellular as well as humoral immune response.

Engineering of recombinant poxviruses is commonly based on homologous DNA recombination. This phenomenon occurs naturally between the viral genomes present within an infected cell during the poxvirus life cycle with a frequency of approximately 0.1% (NAKANO et al., 1982; MACKETT et al., 1984; MOSS, 1996). For the generation of recombinant VACV, homologous recombination is usually directed by a plasmid transfer vector, which contains an expression cassette including a poxvirus-specific promoter next to a multiple cloning site for insertion of foreign genes and a selectable marker (e.g. a fluorescent marker) to facilitate the clonal isolation of recombinant MVA (MACKETT et al., 1984; MOSS, 1996). The virus-specific promoter followed by the foreign gene as well as the marker gene are flanked by poxvirus genomic sequences, which direct the recombination and insertion of heterologous DNA into a desired locus within the poxvirus genome (MACKETT et al., 1984). After infection of the cells with the poxvirus vector followed by transfection with the specific transfer plasmid, homologous recombination occurs and recombinant

viruses can be isolated using the specific selection marker (KREMER et al., 2012).

Recombinant poxviruses engineered for the application as vector vaccines still have the potential to induce life-threatening complications, in particular in immunocompromised individuals, as observed during conventional smallpox vaccination with live VACV (LANE et al., 1969; REDFIELD et al., 1987). Further research of poxviruses in vaccine development focused on replication deficient and attenuated VACV strains. Therefore, non-replicating MVA has been established as viral vector system with an exceptional safety profile due to its replication deficiency in cells of mammalian origin (SUTTER & MOSS, 1992; VOLZ & SUTTER, 2013, 2017). Here, MVA is still able to infect them and to start its molecular life cycle, which is associated with a block in virion assembly that occurs late in viral life cycle. Thus, expression of early as well as late genes is unimpaired, although MVA fails to produce mature virions (SUTTER & MOSS, 1992; VOLZ & SUTTER, 2017). Another attenuated VACV strain is New York attenuated vaccinia virus (NYVAC), which is derived from the VACV strain Copenhagen. But compared to MVA, the life cycle of NYVAC is blocked at an early stage with the result of non-efficient expression of intermediate as well as late genes (TARTAGLIA et al., 1992; PAOLETTI, 1996).

Overall, MVA is characterized by its exceptional safety profile, which is mainly based on the replication deficiency in cells of mammalian origin. The stability of the poxvirus genome and the genetic plasticity allows for the production of high amounts of foreign antigens. In addition, the availability of convenient and well-established laboratory protocols facilitates the generation of recombinant MVAs for large scale production (KREMER et al., 2012).

2.4. MVA interfering with inflammatory immune response

In contrast to conventional VACV, MVA has strong immunostimulating capacities in particular targeting the innate immune system (BLANCHARD et al., 1998; WAIBLER et al., 2007; DELALOYE et al., 2009; HALLE et al., 2009; LEHMANN et al., 2009; ALTENBURG et al., 2014). In general, poxviruses

produce many gene products, which are involved in modulation and evasion of innate immunity (SMITH et al., 2013). About one third of the VACV genes (especially these located at the terminal regions) encodes genes affecting the host immune system (SMITH, 1993). Most of these immunomodulatory proteins are expressed early during poxvirus infection, which enables them to disturb the host innate immune system rapidly (SMITH et al., 2013). The ability of MVA to activate the innate immune system early on can be explained by the significant loss of genetic information, which occurred during serial passages on CEF cells. As a result, many of the poxvirus immunomodulatory genes were depleted in the highly attenuated MVA leading to an innate immune response and induction of adaptive immunity in infected cells (ALTENBURG et al., 2014). Poxviruses have evolved different strategies to interfere with the host innate immune response, i.e. they are able to inhibit cytokine production and other signaling pathways, such as IFN signaling or the apoptotic response (SEET et al., 2003; HAGA & BOWIE, 2005). In terms of IFN response, VACV encodes a large number of proteins, which are able to block the activation of transcription factors NF-κB and IRF-3 (SMITH et al., 2013). In contrast, many of these inhibitors are not present in MVA, such as the proteins A52, B14, C4, C16, K1, M2 and N1. As a result, NF-κB signaling takes place in MVA infected cells and the proteins K1 and M2 could be confirmed as NF-κB inhibitors by re-introduction of corresponding VACV gene sequences into the MVA genome (OIE & PICKUP, 2001; HINTHONG et al., 2008). Another immune evasion strategy of poxviruses is the secretion of viral receptors that are able to bind chemokines and cytokines, such as tumor necrosis factor (TNF), IFN or interleukins (ILs) (ALCAMI & SMITH, 1992, 1995; MOSSMAN et al., 1995; SYMONS et al., 1995; SMITH et al., 1996; GRAHAM et al., 1997; SMITH et al., 2013). Compared to replication competent VACVs, MVA lacks several of these genes encoding receptors that inhibit innate immune response. Blanchard et al. demonstrated that MVA is not able to produce soluble receptors for IFN-α/β, IFN-γ, TNF and some chemokines, but in contrast it is still able to produce a soluble IL-1β receptor (BLANCHARD et al., 1998). IL-1β is a proinflammatory cytokine and it is known to act significantly on the febrile response in VACV and poxvirus infection in general (ALCAMÍ & SMITH, 1996). Immunizations of mice with a MVA deletion mutant defective in IL-1β receptor expression resulted in

increased numbers of virus-specific CD8+ T cells and higher levels of protection against lethal challenge infection with virulent VACV strain Western Reserve (STAIB et al., 2005). Another characteristic of the immunostimulatory properties of MVA is the induction of chemokines, e.g. CCL2, which leads to rapid immigration of leukocytes (LEHMANN et al., 2009; LEHMANN et al., 2015). In addition to the upregulation of chemokines, Price and coworkers demonstrated that MVA is also able to activate the complement system resulting in enhanced migration of leukocytes (PRICE et al., 2015).

The efficacy of vaccine applications largely depends on a high activation of innate and adaptive immune response. Therefore, MVA is highly advantageous for the development of vector vaccines due to its particular ability to rapidly upregulate important host immune responses.

2.5. MVA vaccines in preclinical and clinical trials

Today, MVA serves as one of the most advanced recombinant poxvirus vectors for the development of vaccines against various infectious diseases, e.g. influenza, human immunodeficiency virus (HIV), malaria or tuberculosis, and against cancer (KREIJTZ et al., 2013; VOLZ & SUTTER, 2013; ALTENBURG et al., 2014; SHEEHAN et al., 2015; VOLZ et al., 2015a; SEBASTIAN & GILBERT, 2016). Different recombinant MVAs have been evaluated in both preclinical and clinical trials and they proved to be safe and immunogenic (GILBERT, 2013; GOMEZ et al., 2013).

Ongoing research focuses in particular on the study of MVA-based vector vaccines against different influenza viruses. In 1994, the first recombinant MVA candidate vaccine was generated to deliver simultaneously the influenza virus A/PR/8/34 (H1N1) antigens hemagglutinin (HA) and nucleoprotein (NP). The recombinant MVA (MVA-HA-NP) was shown to be immunogenic in immunized mice. Here, vaccination by different routes with the MVA-HA-NP candidate vaccine resulted in efficient levels of influenza HA-specific antibodies as well as cellular immune responses. Furthermore, mice were protected against lethal respiratory challenge with influenza virus A/PR/8/34 (SUTTER et al., 1994). In

more recent years, a promising recombinant MVA candidate vaccine against highly pathogenic influenza virus H5N1 (MVA-HA-VN/04) was successfully evaluated in different animal models including mice, macaques and chickens (KREIJTZ et al., 2007; VEITS et al., 2008; KREIJTZ et al., 2009a; KREIJTZ et al., 2009b; HESSEL et al., 2011). Of note, MVA-HA-VN/04 was able to induce neutralizing and protective H5-specific antibodies after challenge with homologous and heterologous H5 subtype influenza viruses (KREIJTZ et al., 2007). The promising results of preclinical assessment allowed for phase I/IIa clinical studies of MVA-HA-VN/04 in humans. Here, the candidate vaccine proved to be immunogenic as it efficiently induced high titers of H5-specific antibodies and T cells with cross-reactivity to influenza H5 viruses of other clades (KREIJTZ et al., 2014; DE VRIES et al., 2015; DE VRIES et al., 2018). Another promising MVA-based influenza vaccine candidate targeting the T cell antigens NP and Matrix 1 protein (M1) (MVA-NP-M1) was successfully tested in phase I/IIa clinical trials. In the first clinical trial (phase I), vaccination with MVA-NP-M1 was well-tolerated and immunogenic in healthy adult individuals. Remarkably, this influenza MVA-based vaccine induced significantly higher amounts of responding T cells compared to other influenza vaccines. With regard to the safety record, significantly less local side effects could be observed in intramuscular vaccinated volunteers, compared to the intradermal vaccinated group. However, systemic side effects, such as nausea, vomiting or rigors, were observed in five out of eight individuals, which have received a higher dose of MVA-NP-M1 via the intramuscular route (BERTHOUD et al., 2011). In the second clinical study (phase IIa), the protective capacity of the T cell-based influenza vaccine MVA-NP-M1 was assessed in healthy adults. Given a single intramuscular injection, only two of 11 vaccinated volunteers developed influenza disease after influenza virus challenge infection. Thereby, the number of symptoms was lower than in the control group and also the time of viral shedding was reduced (LILLIE et al., 2012). In the third clinical trial (phase I), MVA-NP-M1 proved to be safe and immunogenic in adults with an age of over 50 years, which present the primary target population for seasonal influenza vaccination. Here, it is noteworthy that older individuals showed similar immune responses to younger volunteers of the former two clinical trials (ANTROBUS et al., 2012).

Well-established protocols allow for the rapid generation of new MVA candidate vaccines. This could be demonstrated in the context of the new emerging pathogen MERS-CoV. Immediately after the discovery of MERS-CoV in 2012, a recombinant MVA expressing the MERS-CoV S protein (MVA-MERS-S) could be generated and successfully evaluated in preclinical studies in mice emphasizing the efficiency and high suitability of MVA as vaccine platform (SONG et al., 2013; VOLZ et al., 2015b). Mice immunized with MVA-MERS-S via different application routes developed virus-neutralizing antibodies as well as MERS-CoV-specific CD8+ T cells leading to protection against MERS-CoV challenge infection (VOLZ et al., 2015b). More recently, the MVA-MERS-S vaccine candidate was evaluated in dromedaries, which are suspected to be responsible for virus spread to humans (HAAGMANS et al., 2014; RAJ et al., 2014a; HAAGMANS et al., 2016). Haagmans and coworkers demonstrated that the vaccine significantly reduced the amount of infectious virus in excretions from MVA-MERS-S immunized dromedary camels after MERS-CoV challenge infection (HAAGMANS et al., 2016). This MVA candidate vaccine is currently undergoing clinical testing in phase I/IIa clinical trial.

In the most recent clinical trial, a MVA-based vector vaccine has been tested against papillomavirus, emphasizing the importance of MVA application in the field of tumor therapy (CABO BELTRAN & ROSALES LEDEZMA, 2019). In this study, single treatment with a MVA vaccine expressing the papillomavirus E2 protein (MVA-E2) eliminated lesions on the vocal cords in 13 patients. The remaining 16 patients showed recurrence of the lesions after single injection. However, the lesions did not return after a second injection with MVA-E2 (CABO BELTRAN & ROSALES LEDEZMA, 2019).

In summary, these highly promising data of preclinical and clinical assessment encourages further development of MVA-based candidate vaccines in the field of human and veterinary medicine and in particular against emerging zoonotic viruses.

3. Cellular immune responses to viral infections

3.1. T cell populations

In basic terms, the immune system is divided into two parts: innate and adaptive immunity (WARRINGTON et al., 2011). Innate immunity represents the first immunological mechanism after exposure to intruding pathogens, which involves rapid and antigen-independent responses (WARRINGTON et al., 2011; LAU & SUN, 2018). Unlike the innate mechanisms of host defense, adaptive immunity is characterized by antigen-specific immune responses and the capacity of immunological memory, which facilitates a more rapid and stronger secondary response when re-exposed to a given pathogen (WARRINGTON et al., 2011; LAU & SUN, 2018; RAPP et al., 2018). The adaptive immunity, is further divided into a cellular and a humoral branch (MURPHY et al., 2008). The cellular part of the adaptive immune system includes T lymphocytes (T cells) and B lymphocytes (B cells), which can differentiate into antibody producing plasma cells (MURPHY et al., 2008; WARRINGTON et al., 2011). As all other blood cells, lymphocytes are derived from pluripotent hematopoietic stem cells in the bone marrow (MURPHY et al., 2008). Those pluripotent stem cells can either differentiate into a common lymphoid progenitor cell or a common myeloid progenitor cell (CHAPLIN, 2010). The myeloid stem cells give rise to erythrocytes, thrombocytes and the other leukocytes, including granulocytes, mast cells and monocytes. Lymphocytes originate from the lymphoid progenitor cell, together with natural killer cells (MURPHY et al., 2008; CHAPLIN, 2010). B cells arise in the bone marrow, whereas T cells require the thymus for maturation (BONILLA & OETTGEN, 2010; LUCKHEERAM et al., 2012). Following differentiation in the primary lymphoid tissues, lymphocytes circulate as so called naïve B and T cells between blood and secondary peripheral lymphoid organs, including lymph nodes and the spleen, until they meet their specific antigen (MURPHY et al., 2008; BONILLA & OETTGEN, 2010).

3.2. T cell functions

Activation of T cells is mediated by antigen-presenting cells. Thereby, a unique T cell receptor expressed on the surface of T cells recognizes a specific antigen fragment bound to a cell-surface protein, known as major histocompatibility complex (MHC), presented by antigen-presenting cells (WARRINGTON et al., 2011; GAUD et al., 2018). The development of functional T cell receptors is regulated by positive and negative selection during differentiation process in the thymus. T cells carrying a T cell receptor, which is able to bind with low avidity to self-MHC complexed with self-antigens undergo positive selection. In contrast, negative selection occurs when T cell receptors bind with very high avidity to self-antigens leading to deletion of such self-reactive T cell clones (KLEIN et al., 2009; BONILLA & OETTGEN, 2010). Coordinated series of genomic rearrangements, known as somatic recombination, lead to the tremendously high diversity of T cell receptors, each with unique specificity for a different antigen (BONILLA & OETTGEN, 2010; CHAPLIN, 2010). Remarkably, about 30,000 T cell receptors are expressed on the surface of one T lymphocyte (MURPHY et al., 2008). T cell receptors are heterodimeric molecules composed of two transmembrane polypeptides termed as α and β chain, which are linked by a disulfide bond (MURPHY et al., 2008; GAUD et al., 2018). Both chains consist of a variable domain (Vα, Vβ), which confer binding to a specific MHC/peptide complex, and a constant domain (Cα, Cβ) anchored into the plasma membrane (MURPHY et al., 2008). The T cell receptor αβ-heterodimer is associated with a signal-transducing CD3 complex, including CD3γ, CD3δ and CD3ε subunits (GAUD et al., 2018). T lymphocytes further express CD4 and CD8 co-receptors on their surface, leading to the classification of CD4+ and CD8+ T cells (MURPHY et al., 2008). These co-receptors bind to conserved regions in the MHC molecules and stabilize the interaction between T cell receptor and MHC/antigen complex (BONILLA & OETTGEN, 2010; GAUD et al., 2018). CD8+ T cells are mainly involved in the defense of viruses by inducing apoptosis of virus-infected cells. Thus, CD8+ T cells are also called cytotoxic T cells (MURPHY et al., 2008). CD4+ T cells, also known as T helper cells (T_H cells), present the major part of T cells and they are responsible for the defense of extracellular bacteria and parasites (BONILLA &

OETTGEN, 2010; LUCKHEERAM et al., 2012). Furthermore, they play a major role in mediating the immune response, including the secretion of cytokines, the activation of other immune cells, such as macrophages and B lymphocytes, and the suppression of autoimmune reactions (LUCKHEERAM et al., 2012). After being activated, CD4+ T cells differentiate into two main subtypes, T_H1 and T_H2 cells (MURPHY et al., 2008).

3.3. T cell activation and antigen-presenting pathways

As described above, T cells are activated by interaction of their specific receptor with antigenic peptide fragments bound to MHC molecules on the surface of antigen-presenting cells (BONILLA & OETTGEN, 2010; WARRINGTON et al., 2011). MHC molecules are cell surface glycoproteins, which are classified as either class I or class II molecules (CHAPLIN, 2010; WARRINGTON et al., 2011). MHC molecules are encoded by a large group of genes, which is named major histocompatibility complex (MURPHY et al., 2008). MHC-class I expression is constitutive in all nucleated cells, whereas MHC-class II molecules are only present on cells of the immune system, including macrophages, dendritic cells and B cells (BONILLA & OETTGEN, 2010; WARRINGTON et al., 2011). MHC-class I molecules are comprised of a polymorphic transmembrane α chain and a non-covalently attached β_2-microglobulin (CHAPLIN, 2010). The larger α chain is subdivided into three domains, α_1, α_2, and α_3, whereby subunit α_1 and α_2 build the peptide- binding site and subunit α_3 is responsible for membrane anchoring (MURPHY et al., 2008). MHC-class II molecules also consist of two non-covalently attached polypeptide chains α and β, which are both divided into two subunits, α_1/α_2 and β_1/β_2 (MURPHY et al., 2008). Here, the antigen-binding site is formed by α_1 and β_1, whereas α_2 and β_2 are anchored into the plasma membrane (MURPHY et al., 2008). Priming of CD8+ T cells is mediated by interaction with antigenic peptides bound to MHC-class I molecules. These MHC-class I-restricted peptides originate from cytosolic proteins, which are degraded in the proteasome and encoded by intracellular replicating pathogens like viruses and some bacteria (endogenous pathway) (BONILLA & OETTGEN, 2010). In

contrast, CD4+ T cells recognize peptides displayed on MHC-class II molecules. MHC-class II-restricted peptides are derived from exogenous pathogens, which are incorporated into antigen-presenting cells via phagocytosis or endocytosis (exogenous pathway) (BLUM et al., 2013). Extracellular pathogens are recognized by pattern recognition receptors (PRRs), such as toll-like receptors, expressed on the surface of dendritic cells, macrophages and other cells (LUCKHEERAM et al., 2012; IWASAKI & MEDZHITOV, 2015). PRRs are specialized for the detection of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides of the bacterial cell wall (TAKEDA et al., 2003; IWASAKI & MEDZHITOV, 2004). After recognition, extracellular pathogens are incorporated via phagocytosis and antigenic peptides are generated by proteolytic processing in the lysosomes for presenting on MHC-class II molecules (BLUM et al., 2013). Dendritic cells are considered to play a crucial role in exogenous antigen presentation and the corresponding activation of CD4+ T cells (JENKINS et al., 2001; LUCKHEERAM et al., 2012). Furthermore, they are essential for a process called cross-presentation, whereby exogenous antigens are processed into the MHC-class I pathway (ROCK & SHEN, 2005). This process facilitates the generation of immunity against viruses, which are able to suppress antigen processing through the endogenous pathway or only infect hematopoietic cells (SIGAL et al., 1999; CHAPLIN, 2010).

T cell-mediated immune response is characterized by three different phases: clonal expansion, contraction and the formation of long-lived memory (WILLIAMS & BEVAN, 2007; RAPP et al., 2018). After initial exposure to a specific antigen, T lymphocytes start to differentiate into effector T cells and undergo massive proliferation (CHAPLIN, 2010; POLONSKY et al., 2016). This process, called clonal expansion, is followed by apoptosis of most of the effector T cells leading to contraction of the expanded T lymphocytes (RAPP et al., 2018). This small fraction of T cells is maintained as long-lived memory T cells mediating immunological memory (RAPP et al., 2018). Immunological memory is defined as the ability of the immune system to produce a more rapid or robust secondary response when re-exposed to a given pathogen (LAU & SUN, 2018; RAPP et al., 2018). The formation of long-lived immunological memory constitutes the basis for efficient and protective vaccination

(SALLUSTO et al., 2010).

3.4. Methods for T cell monitoring

Enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICS) detecting IFN-γ secretion are the most commonly used assays for quantifying and characterizing T cell-mediated immune response in natural infections as well as vaccination studies (CURRIER et al., 2002; STREECK et al., 2009; WU et al., 2012; FIORE-GARTLAND et al., 2016; MALM et al., 2016). IFN-γ is produced by cytotoxic CD8+ as well as T_H1 CD4+ effector cells, and this cytokine plays a crucial role in immunity against intracellular pathogens, such as viruses (SCHOENBORN & WILSON, 2007).

The ELISPOT assay is based on the principle of an enzyme-linked immunosorbent assay (ELISA). The frequency of cytokine-secreting T cells is measured after *ex vivo* stimulation with one or multiple peptides (FIORE-GARTLAND et al., 2016). IFN-γ molecules secreted from activated T cells are captured by an immobilized antibody and visualized by an enzyme-linked secondary antibody (ANTHONY & LEHMANN, 2003; STREECK et al., 2009). One of the main advantages of the ELISPOT assay is the ability to rapidly and efficiently screen a wide array of peptide antigens allowing the detection of T cell specificities to an entire vaccine immunogen (ANTHONY & LEHMANN, 2003; FIORE-GARTLAND et al., 2016). Mapping of antigen-specific epitopes is facilitated by the use of overlapping peptides to cover every possible determinant within a given antigenic sequence (ANTHONY & LEHMANN, 2003). Moreover, the use of peptide pools allows the reduction of assays to identify T cell epitope specificities within the peptide array (ANTHONY & LEHMANN, 2003; FIORE-GARTLAND et al., 2016). Although ELISPOT assay is highly sensitive, specific cell types, which contribute to IFN-γ production, cannot be identified (TOBERY et al., 2006; MALM et al., 2016). In that context, ICS has proved to be a valuable tool for enumerating and differentiation of vaccine- and infection-induced T cells (DE ROSA, 2012). ICS is based on the flow cytometry technique. Here, stimulated cytokine-producing cells are cultured in the presence of a protein secretion inhibitor, which leads to the accumulation

of the cytokines within the antigen-specific cell. After stimulating, the cells are fixed and permeabilized followed by staining of intracellular cytokines and cell markers with fluorescence-conjugated antibodies (FREER & RINDI, 2013). Compared to the ELISPOT assay, ICS assays are less sensitive. However, the main advantage of this assay is the ability to measure multiple cytokines or T cell functions and phenotypes (DE ROSA, 2012; FREER & RINDI, 2013). This feature allows for qualitative analysis of activated T cells including the differentiation of cell subpopulations responsible for cytokine production (WU et al., 2012; FREER & RINDI, 2013; MALM et al., 2016). The quantification of antigen-specific T cells and the determination of their function and phenotype are essential to measure immunogenicity in studies of infectious diseases and during vaccine trials.

III. OBJECTIVES

In terms of ongoing epidemics caused by MERS-CoV and due to the lack of approved vaccines or therapies for MERS, this work describes the following:

(i) the generation of recombinant MVA expressing the MERS-CoV N protein (MVA-MERS-N)

(ii) *in vitro* characterization of recombinant MVA-MERS-N

- genetic analysis at the DNA level
- analysis of protein expression
- replication analysis

(iii) *in vivo* characterization of cellular immune responses recognizing the MERS-CoV N protein

- immunization experiments in mice
- analysis of MVA-MERS-N-induced T cell responses using a two-dimensional, pooled-peptide matrix system

IV. RESULTS

The manuscript is presented in form accepted for publication (VEIT et al., 2018).

CD8+ T Cells Responding to the Middle East Respiratory Syndrome Coronavirus Nucleocapsid Protein Delivered by Vaccinia Virus MVA in Mice

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Middle East respiratory syndrome coronavirus (MERS-CoV), a novel infectious agent causing severe respiratory disease and death in humans, was first described in 2012. Antibodies directed against the MERS-CoV spike (S) protein are thought to play a major role in controlling MERS-CoV infection and in mediating vaccine-induced protective immunity. In contrast, relatively little is known about the role of T cell responses and the antigenic targets of MERS-CoV that are recognized by CD8+ T cells. In this study, the highly conserved MERS-CoV nucleocapsid (N) protein served as a target immunogen to elicit MERS-CoV-specific cellular immune responses. Modified Vaccinia virus Ankara (MVA), a safety-tested strain of vaccinia virus for preclinical and clinical vaccine research, was used for generating MVA-MERS-N expressing recombinant N protein. Overlapping peptides spanning the whole MERS-CoV N polypeptide were used to identify major histocompatibility complex class I/II-restricted T cell responses in BALB/c mice immunized with MVA-MERS-N. We have identified a H2-d restricted decamer peptide epitope in the MERS-N protein with CD8+ T cell antigenicity. The identification of this epitope, and the availability of the MVA-MERS-N candidate vaccine, will help to evaluate MERS-N-specific immune responses and the potential immune correlates of vaccine-mediated protection in the appropriate murine models of MERS-CoV infection.

Introduction

The Middle East respiratory syndrome coronavirus (MERS-CoV), a hitherto unknown β-coronavirus, emerged as a causative agent of a severe respiratory disease in humans in 2012. This new coronavirus was first isolated from the sputum of a patient suffering from severe pneumonia and renal failure (ZAKI et al., 2012). To date, the MERS-CoV still causes disease and death in humans with a total of 2260 confirmed cases including 803 fatalities (PARK et al., 2018; WORLD HEALTH ORGANIZATION (WHO), 2019b, 2019a). Epidemiological data suggest that the MERS-CoV is endemic in Saudi Arabia, which accounts for the majority of primary community-acquired cases. Many of those primary cases are due to virus exposure through direct contact with dromedary camels, the primary animal reservoir of MERS-CoV. Alternatively, camel workers undergoing subclinical infections are suggested to mediate virus transmission to

other susceptible individuals (ALRADDADI et al., 2016; ALSHUKAIRI et al., 2018). Other outbreaks of MERS have been caused by nosocomial transmissions in health care settings (ASSIRI et al., 2013b; KI, 2015; OH et al., 2018; AL-TAWFIQ & AUWAERTER, 2019). Most of the MERS-CoV infections occur within the Arabian Peninsula, i.e., Saudi Arabia, Qatar, and United Arab Emirates, however MERS cases have been reported in various other countries around the world (KRAAIJ-DIRKZWAGER et al., 2014; OH et al., 2018). At present, there is still relatively little known about the pathogenesis of MERS-CoV. The highest incidence of severe disease is observed in elderly and immunocompromised individuals (ASSIRI et al., 2013a). Those at general risk for infections are health care workers and people in close contact with dromedary camels (AL-TAWFIQ & MEMISH, 2014; MEMISH et al., 2014). These groups are therefore considered relevant target populations for prophylactic vaccination against MERS-CoV infection and prevention of MERS. The World Health Organization (WHO) and the Coalition for Epidemic Preparedness Innovations (CEPI) have listed MERS as priority target for vaccine development (ROTTINGEN et al., 2017). However, so far, no candidate vaccines have proceeded beyond phase I/IIa clinical testing (CHO et al., 2018). One of these candidate vaccines is based on Modified Vaccinia virus Ankara (MVA), a safety-tested and replication-deficient vaccinia virus serving as an advanced viral vector platform for the development of new vaccines against infectious diseases and cancer (for review see (VOLZ & SUTTER, 2017)). In that context, we still know relatively little about the correlates of vaccine induced protection against MERS-CoV. It is well-known that virus-neutralizing antibodies directed against the spike glycoprotein (S protein) correlate with protective immunity against coronavirus infections in general (SUNE et al., 1991; KOLB et al., 2001; BISHT et al., 2004; YANG et al., 2004). Since the S protein is present on the cell surface, S protein is considered as the major antigen to induce virus neutralizing antibodies and as a key immunogen for the development of MERS-CoV candidate vaccines (SONG et al., 2013; MALCZYK et al., 2015; MUTHUMANI et al., 2015; VOLZ et al., 2015b; WIRBLICH et al., 2017). However, based on current knowledge from the biology of β-coronaviruses, we hypothesize that also other viral proteins warrant consideration as immunogens and targets of virus-specific antibodies and T cells. Among those, the

nucleocapsid protein (N protein) is produced at high levels in infected cells and has been proposed as useful candidate protein for clinical diagnosis (LAU et al., 2004; TIMANI et al., 2004; HE et al., 2005; CHEN et al., 2015; YAMAOKA et al., 2016). The coronavirus N proteins have been associated with multiple functions in the virus life cycle including the regulation of viral RNA synthesis, the packaging of the viral RNA in helical nucleocapsids, and in virion assembly through interaction with the viral M protein (ALMAZAN et al., 2004; ZUNIGA et al., 2010; MCBRIDE et al., 2014; HSIN et al., 2018). Furthermore, several reports suggest that the severe acute respiratory syndrome coronavirus (SARS-CoV) N protein functions as an immune evasion protein and an antagonist of the host interferon response (SPIEGEL et al., 2005; KOPECKY-BROMBERG et al., 2007; LU et al., 2011). Recently, the overexpression of MERS-CoV N in human A549 cells was found to be linked to an up-regulation of antiviral host gene expression including the synthesis of the inflammatory chemokine CXCL10 (ABOAGYE et al., 2018). Despite this possible immune modulatory activity, SARS-CoV N-specific immune responses are reported to be long-lived and more broadly reactive when compared to SARS-CoV S-specific immunity (TANG et al., 2011). Likewise, we were curious as to the suitability of the MERS-CoV N protein to serve as a vaccine antigen. The N protein is not present on the surface of MERS-CoV particles nor is it predicted to be expressed on the surface membrane of MVA infected cells. From this we hypothesized that the most relevant part of MERS-CoV N-specific immunity is based on CD8+ T cell responses relying on the processing and presentation of intracellular antigens. Currently, there is little information about MERS-CoV N-specific immune responses including the in vivo induction of N-specific cellular immunity.

In this study, we investigated the synthesis and delivery of the MERS-CoV N protein as a privileged antigen by a MVA vector virus. The recombinant MVA expressing a synthetic gene sequence of full-length MERS-CoV N (MVA-MERS-N) proved genetically stable and fully replication-competent in chicken embryo fibroblasts, an established cell substrate for MVA vaccine manufacturing. Upon in vitro infection, MVA-MERS-N produced high amounts of the heterologous protein that were detectable with MERS-CoV N-specific antibodies. Furthermore, MVA-MERS-N was tested as an experimental vaccine

in BALB/c mice and elicited MERS-CoV N-specific interferon γ (IFN-γ)-producing CD8+ T cells. Using peptide library covering the whole MERS-CoV N polypeptide, we identified new H2-d restricted peptide epitopes of MERS-CoV N in BALB/c mice. This data will be highly relevant for further assessment of N antigen-specific immune responses in the well-established MERS-CoV-BALB/c mouse immunization/challenge model (ZHAO et al., 2014; CHI et al., 2017; COLEMAN et al., 2017; LIU et al., 2017; JUNG et al., 2018).

Materials and Methods

Mice

Female BALB/c mice (6 to 10-week-old) were purchased from Charles River Laboratories (Sulzfeld, Germany). For experimental work, mice were housed in an isolated (ISO) cage unit (Tecniplast, Hohenpeißenberg, Germany) and had free access to food and water. All animal experiments were handled in compliance with the German regulations for animal experimentation (Animal Welfare Act, approved by the Government of Upper Bavaria, Munich, Germany).

Cells

Primary chicken embryo fibroblasts (CEF) were prepared from 10-day-old chicken embryos (SPF eggs, VALO, Cuxhaven, Germany) and maintained in Minimum Essential Medium Eagle (MEM) (SIGMA-ALDRICH, Taufkirchen, Germany) containing 10% heat-inactivated fetal bovine serum (FBS) (SIGMA-ALDRICH, Taufkirchen, Germany), 1% Penicillin-Streptomycin (SIGMA-ALDRICH, Taufkirchen, Germany), and 1% MEM non-essential amino acid solution (SIGMA-ALDRICH, Taufkirchen, Germany). Human HeLa (ATCC CCL-2) cells were maintained in MEM containing 10% FBS and 1% Penicillin-Streptomycin. Human HaCat (CLS Cell Lines Service GmbH, Eppelheim, Germany) cells were cultured in Dulbecco`s Modified Eagle`s Medium (SIGMA-ALDRICH, Taufkirchen, Germany) supplemented with 10% heat-inactivated FBS, 2% HEPES-solution (SIGMA-ALDRICH, Taufkirchen, Germany) and antibiotics as described above. All cells were maintained at 37 °C and 5% CO_2 atmosphere.

Plasmid Constructions

The cDNA encoding the entire amino acid (aa) sequence (413 aa) of the MERS-CoV N protein was in silico modified by introducing silent codon alterations to remove three termination signals (TTTTTNT) for vaccinia virus early transcription and two G/C nucleotide runs from the original MERS-CoV gene sequence (Human betacoronavirus 2c EMC/2012, GenBank accession no. JX869059). A cDNA fragment was generated by DNA synthesis (Invitrogen Life Technology, Regensburg, Germany) and cloned into the MVA transfer plasmid pIIIH5red (KREMER et al., 2012) to place the MERS-CoV N gene sequence under the transcriptional control of the vaccinia virus early/late promoter PmH5 (WYATT et al., 1996) resulting in the MVA vector plasmid pIIIH5red-MERS-N.

Generation of Recombinant Virus

Recombinant MVA was generated using standard methodology as described previously (KREMER et al., 2012). Briefly, monolayers of nearly confluent CEF grown in six-well tissue culture plates (Sarstedt, Nürnbrecht, Germany) were infected with non-recombinant MVA (clonal isolate MVA F6) at 0.05 multiplicity of infection (MOI) and, 45 min after infection, CEF cells were transfected with plasmid pIIIH5red-MERS-N DNA using X-tremeGENE DNA Transfection Reagent Lipofectamine (Roche Diagnostics, Penzberg, Germany) as recommended by the manufacturer. At 48 h after infection, the cell cultures were harvested and recombinant MVA expressing the MERS-CoV N protein was clonally isolated by consecutive rounds of plaque purification in CEF by screening for transient co-expression of the red fluorescent marker protein mCherry.

Quality control experiments were performed using standard methodology (KREMER et al., 2012). Genetic identity and genetic stability of the recombinant virus were assessed via polymerase chain reaction (PCR) analysis of the genomic viral DNA. Replicative capacities of the MVA vector virus was tested in multi-step growth experiments in CEF and human HaCat and HeLa cells.

To generate high titer vaccine preparations for preclinical studies, recombinant MVA was amplified in CEF, purified by ultracentrifugation through 36% sucrose

cushions, resuspended in 10 mM Tris-HCl buffer, pH 9.0, and stored at −80 °C. The sucrose purified MVA-MERS-N vaccine preparations corresponded in total protein/total DNA content to the purity profile of a MVA candidate vaccine for human use.

Western Blot Analysis of Recombinant Proteins

Confluent cell monolayers of CEF or HaCat cells were infected at a MOI of 5 with recombinant MVA expressing the MERS-CoV N or S protein (SONG et al., 2013). Non-infected (mock) or wild-type MVA-infected cells served as controls. Cell lysates were prepared at different time points after infection and stored at −80 °C. Total cell proteins were resolved by electrophoresis in a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel (SDS-PAGE) and subsequently transferred onto a nitrocellulose membrane via electroblotting. After 1 h blocking in a phosphate buffered saline (PBS) buffer containing 1% (w/v) non-fat dried milk and 0.1% (v/v) NP-40 detergent, the blots were incubated with monoclonal mouse anti-MERS-CoV Nucleocapsid antibody (Sino Biological, Beijing, China, 1:1000), monoclonal rabbit anti-MERS-CoV Spike Protein S1 Antibody (Sino Biological, 1:500), or polyclonal sera from MERS-CoV infected rabbits or cynomolgus macaques (kindly provided by Dr. Bart Haagmans, Erasmus Medical Center, Rotterdam, 1:1000) (SONG et al., 2013) as primary antibodies. After washing with 0.1% NP-40 in PBS, the blots were incubated with anti-mouse IgG (1:5000), or anti-rabbit IgG antibody (1:5000), or protein A (1:1000) conjugated to horseradish peroxidase (Cell Signaling Technology, Frankfurt am Main, Germany). After further washing, blots were developed using SuperSignal® West Dura Extended Duration substrate (Thermo Fisher Scientific, Planegg, Gemany).

Immunization Experiments in Mice

Groups of female BALB/c mice (n = 2 to 5) were immunized twice within a 21-day interval with 10^8 plaque-forming-units (PFU) of recombinant MVA-MERS-N or non-recombinant MVA (MVA) or PBS as mock vaccine. Vaccinations were given via the intramuscular (i.m.) or intraperitoneal (i.p.) route using 25 µL (i.m.) or 200 µL (i.p.) volumes per inoculation. All mice were monitored daily for welfare and potential adverse events of immunization. At day 8 post prime-boost immunization, animals were sacrificed by cervical dislocation and spleens

were taken for T cell analysis.

Synthetic Peptides and Design of Peptide Pools

For T cell immune monitoring, we identified 101 individual synthetic peptides (assigned as 1 to 101) in silico spanning the entire MERS-CoV N protein sequence (Human betacoronavirus 2c EMC/2012, GenBank accession no. JX869059). This peptide library was designed to contain 15-mer peptides overlapping by 11 aa. Eighty-four peptides could be synthesized (Thermo Fisher Scientific) and were organized into two-dimensional matrix peptide pools (V1 to V9 and H1 to H9) containing 9 or 10 peptides as described previously (FIORE-GARTLAND et al., 2016; MALM et al., 2016). For further T cell epitope mapping, the 11 aa sequence shared between peptide #89 and #90 was trimmed into 8-10-mer peptides, which were also obtained from Thermo Fisher Scientific. All peptides were dissolved in PBS to a concentration of 2 mg/mL and stored at −20 °C until use.

T cell Analysis by Enzyme-Linked Immunospot (ELISPOT)

Spleens were harvested on day 8 post prime-boost vaccination. Splenocytes were prepared by passing through a 70 µm strainer (Falcon®, A Corning Brand, Corning, USA) and incubating with Red Blood Cell Lysis Buffer (SIGMA-ALDRICH, Taufkirchen, Germany). Cells were washed and resuspended in RPMI 1640 medium (SIGMA-ALDRICH) containing 10% heat inactivated FBS and 1% Penicillin-Streptomycin. Splenocytes were further processed by using the QuadroMACS Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) to separate CD8+ and CD4+ splenocytes with MACS Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

IFN-γ-producing T cells were measured using ELISPOT assays (Elispot kit for mouse IFN-γ, MABTECH, Germany) following the manufacturer`s instructions. Briefly, $1x10^6$ splenocytes were seeded in 96-well plates (Sarstedt, Nürnbrecht, Germany) and stimulated with peptide pools or individual peptides (2 µg peptide/mL RPMI 1640 medium) at 37 °C for 48 h. Non-stimulated cells and cells treated with phorbol myristate acetate (PMA) (SIGMA-ALDRICH) and ionomycin (SIGMA-ALDRICH, Taufkirchen, Germany) or MVA $F2L_{26-34}$ peptide (F2L, SPGAAGYDL, Thermo Fisher Scientific, Planegg, Germany) (TSCHARKE

et al., 2006) served as negative and positive controls, respectively. Automated ELISPOT plate reader software (A.EL.VIS Eli.Scan, A.EL.VIS ELISPOT Analysis Software, Hannover, Germany) was used to count and analyze spots.

T Cell Analysis by Intracellular Cytokine Staining (ICS) and Flow Cytometry

Splenocytes were prepared as described above. Splenocytes were added to 96-well plates ($1x10^6$ cells/well) and stimulated for 6 h with MERS-CoV N-specific peptide (at 8 µg peptide/mL RPMI 1640 medium) in presence of the protein transport inhibitor Brefeldin A (Biolegend, San Diego, CA, USA; 5 µg/mL). Non-stimulated cells served as a background control and cells stimulated with 5 ng/mL PMA and 500 ng/mL ionomycin or with F2L peptide (8 µg/mL RPMI 1640 medium) were used as positive controls. After stimulation, cell surface antigens were stained using PE-conjugated anti-mouse CD3 (clone: 17A2, Biolegend, San Diego, CA, USA), PE/Cy7-conjugated anti-mouse CD4 (clone: GK1.5, Biolegend, San Diego, CA, USA), or FITC-conjugated anti-mouse CD8a (clone: 5H10-1, Biolegend, San Diego, CA, USA) antibody and incubated for 30 min on ice. The surface-stained cells were washed with staining buffer (MACS QuantTM Running Buffer, Miltenyi Biotec), then fixed and permeabilized with Fixation- and Perm/Wash-Buffer (Biolegend, San Diego, CA, USA), and finally stained for intracellular IFN-γ expression using APC-conjugated anti-mouse-IFN-γ antibody (clone: XMG1.2, Biolegend, San Diego, CA, USA) for 30 min on ice. Following final washes, cells were resuspended in staining buffer and analyzed using the MACS Quant® VYB flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany).

Statistical Analysis

Statistical analysis was performed by t-test using GraphPad Prism version 5 software (GraphPad software, San Diego CA, USA); P-values less than 0.05 were considered to be statistically significant.

Results

Construction and Characterization of Recombinant MVA Expressing MERS-CoV N Gene Encoding Sequences

Recombinant virus MVA-MERS-N was formed in CEF that were infected with MVA and transfected with the MVA vector plasmid pIIIH5red-MERS-N (Figure 1a). The MVA DNA sequences in pIIIH5red-MERS-N (flank-1, flank-2) targeted the insertion of the N gene sequences into the site of deletion III within the MVA genome. The clonal isolation was facilitated by co-production of the red fluorescent reporter protein mCherry allowing for the convenient detection of MVA-MERS-N infected cells during plaque purification. The repetitive DNA sequence of flank-1 (FR) served to remove the marker gene mCherry from the genome of the final recombinant virus through initiating an intragenomic homologous recombination (marker gene deletion). After PCR analysis confirmed the presence of more than 95% MVA-MERS-N recombinant viruses in the cultures, we selected the final marker-free recombinant viruses by plaque purification and screening for plaques without mCherry fluorescence. To confirm genetic integrity and proper insertion of the heterologous N gene sequences within the MVA-MERS-N genome, we analyzed viral genomic DNA by PCR using specific oligonucleotide primers specific for MVA sequences adjacent to the deletion III insertion site (Figure 1b). Additional PCRs specific for MVA sequences within the C7L gene locus or adjacent to the major deletion sites I, II, IV, V, and VI served to control for the genetic identity and genomic stability of MVA-MERS-N (Figure 1c, and data not shown). Next, we evaluated the recombinant virus MVA-MERS-N by multi-step growth analysis in different cell lines (Figure 1d). In CEF, the cell culture routinely used to propagate recombinant MVA vaccines, MVA-MERS-N efficiently replicated to titers similar to those obtained with non-recombinant MVA. In contrast, the human cell lines HaCat and HeLa proved non-permissive for productive virus growth confirming the well-preserved replication deficiency of the recombinant MVA-MERS-N in cells of mammalian origin.

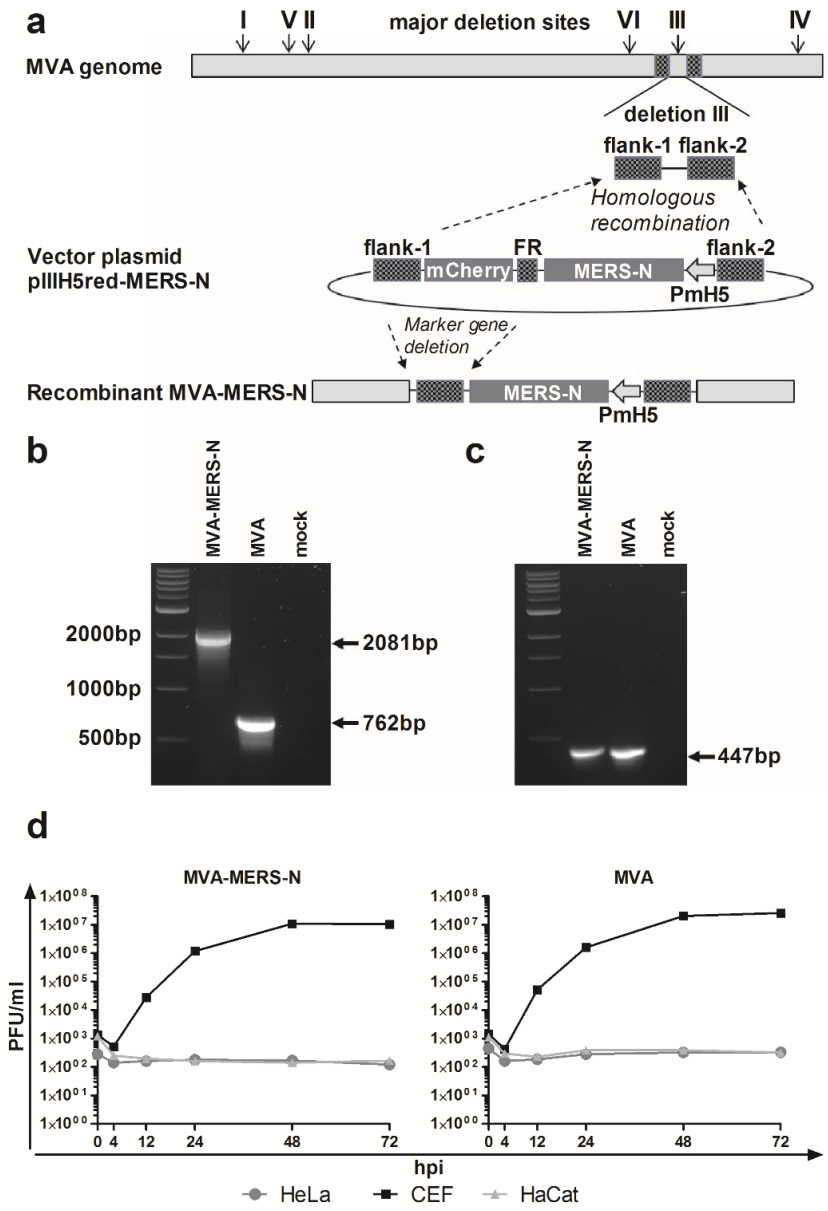

Figure 1. Generation and characterization of recombinant Modified Vaccinia virus Ankara expressing the Middle East respiratory syndrome coronavirus N protein (MVA-MERS-N); (**a**) Schematic diagram of the MVA genome indicating the major deletion sites I-VI on the top. Flank-1 and flank-2 refer to MVA DNA sequences adjacent to corresponding insertion site. Deletion III was used to insert MERS-N encoding gene sequences under the transcriptional control of the vaccinia virus promoter PmH5. Repetitive sequences (FR) were designed to remove the mCherry marker by intragenomic homologous recombination (marker gene deletion); (**b–c**) PCR analyses of genomic viral DNA using oligonucleotide primers to confirm the correct insertion of recombinant MERS-N gene into deletion III (**b**), and the genetic integrity of the MVA genome for the C7L gene locus (**c**); (**d**) Multi-step growth analysis of recombinant MVA-MERS-N and non-recombinant MVA (MVA); Chicken embryo fibroblasts (CEF) and human HaCat or HeLa cells were infected at a multiplicity of infection (MOI) of 0.05 with MVA-MERS-N or MVA. Infected cells were collected at different time points after infection and titrated on CEF cells.

To confirm the synthesis of MERS-CoV N protein upon MVA-MERS-N infection, total cell proteins from infected CEF and HaCat cells were separated by SDS-PAGE and analyzed by immunoblotting (Figure 2). Consistent with the expected molecular mass of the MERS-CoV N protein we readily detected a ~45 kDa polypeptide using the N-specific mouse monoclonal antibody. At 24 hours post infection (hpi) a prominent band of N protein was visible in the lysates from both cell lines suggesting efficient synthesis of the recombinant protein under permissive and non-permissive growth conditions for MVA-MERS-N (Figure 2a).

In addition, the comparative Western blot analysis of cell lysates from MVA-MERS-N or MVA-MERS-S infected CEF with antigen-specific mouse monoclonal antibodies suggested the production of comparable amounts of both MERS-CoV candidate antigens (Figure 2b). This observation is in line with the fact that the MVA-MERS-S candidate vector vaccine expresses the MERS-CoV S gene sequences using the identical PmH5 promoter system (SONG et al., 2013). As shown in previous studies, we detected two MERS-CoV S-specific protein bands upon infection with MVA-MERS-S indicating the authentic proteolytic cleavage of the full-length S glycoprotein (~210 kDa) into an N-terminal (~120 kDa S1 domain) and a C-terminal (~85 kDa S2 domain; not detected) subunit (GIERER et al., 2013; SONG et al., 2013; MILLET & WHITTAKER, 2014). Following this, we used the total protein lysates from MVA-MERS-N or MVA-MERS-S infected CEF to assess the recognition of the MERS-CoV N and S antigens by sera from experimentally MERS-CoV infected animals. The Western blot analysis of sera from an infected rabbit (Figure 2c) or a cynomolgus monkey (Figure 2d) revealed the presence of antibodies specific for the MERS-CoV N protein. The recognition of the MERS-CoV N protein was at least as prominent as the MERS-CoV S antigen, which was suggestive of the induction of substantial N-specific antibody responses after experimental MERS-CoV infections.

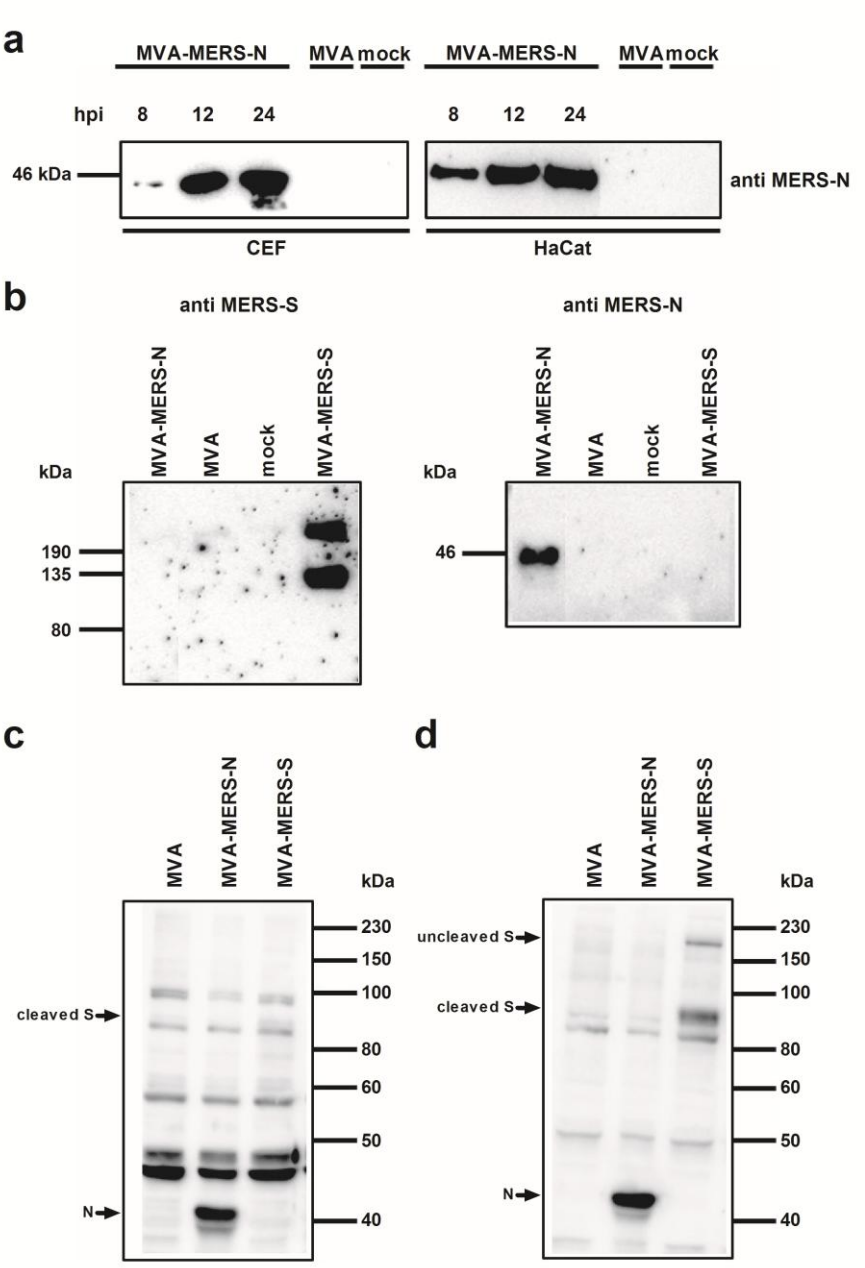

Figure 2. Analysis of recombinant MVA-MERS proteins; (**a**) Western Blot analysis of MERS-CoV N protein produced in CEF or HaCat cells. Lysates from cells infected with recombinant MVA (MVA-MERS-N, MVA-MERS-S) or non-recombinant MVA (MVA) at a MOI of five, or from non-infected cells (mock) were prepared at eight, 12, or 24 hpi. Proteins were analyzed by immunoblotting with a monoclonal anti-MERS-N antibody; (**b–d**) Western Blot analysis of MERS-CoV N and S proteins produced in CEF. Total cell extracts from CEF infected with recombinant MVA (MVA-MERS-N, MVA-MERS-S) or non-recombinant MVA (MVA) at a MOI of five, or from non-infected cells (mock) were prepared at 24 hpi. Cell lysates and proteins were tested by immunoblotting using monoclonal anti MERS-N and anti MERS-S antibody (**b**) or polyclonal sera from MERS-CoV infected rabbits (**c**) or cynomolgus macaques (**d**). Arrows indicate the N- or S-specific protein bands.

Characterization of MERS-CoV N-specific T Cell Responses

Initial Screen of MERS-CoV N Epitopes Using Overlapping Peptide Pools

T cell responses against coronaviruses are known to be long lived and mostly target the more conserved CoV internal structural N protein. However, information on MERS-CoV N antigen-specific T cell specificities is still limited. Thus, we aimed first to identify N polypeptide-specific T cell epitopes in BALB/c mice immunized twice with recombinant virus MVA-MERS-N or non-recombinant MVA as a control via the intraperitoneal and intramuscular routes. Eight days after the final immunization, splenocytes were prepared and the purified CD4+ and CD8+ T cells were restimulated in vitro with overlapping peptides corresponding to the N protein. Overlapping peptides were pooled using a two-dimensional, pooled-peptide matrix system (Table S1) and screened by IFN-γ ELISPOT.

Table S1. The design of matrix peptide pools for systematically screening of H2-d restricted T cell epitopes in MERS-CoV N protein.

Matrix	Pool	Peptide								
1	H1	2	3	4	6	7	8	9	10	11
	H2	12	13	14	15	16	17	18	19	22
	H3	23	24	25	26	27	28	29	30	31
	H4	32	33	34	35	36	37	38	39	49
	H5	50	51	52	53	54	55	56	57	58
	H6	59	60	65	66	67	68	69	70	71
	H7	72	73	74	75	76	77	78/79	80	81
	H8	82	83	84	85	86	87	88	89/90	91
	H9	92	93	94	95	96	97	98	99	100/101
2	V1	2	12	23	32	50	59	72	82	92
	V2	3	13	24	33	51	60	73	83	93
	V3	4	14	25	34	52	65	74	84	94
	V4	6	15	26	35	53	66	75	85	95
	V5	7	16	27	36	54	67	76	86	96
	V6	8	17	28	37	55	68	77	87	97
	V7	9	18	29	38	56	69	78/79	88	98
	V8	10	19	30	39	57	70	80	89/90	99
	V9	11	22	31	49	58	71	81	91	100/101

The stimulation of splenoctyes from MVA-MERS-N immunized mice with the peptides from 16 out of the total 18 peptide pools did not result in the detection of IFN-γ producing T cells above background numbers obtained with splenocytes from mock or MVA-control vaccinated animals. Stimulation with the peptides from pools H8 (n = 10) and V8 (n = 10) as well as the use of the

vaccinia virus positive peptide F2L (TSCHARKE et al., 2006) (data not shown) showed elevated numbers of IFN-γ spot forming cells (SFC) in CD8+ T cell cultures (Figure 3a,b). MVA-MERS-N immunizations given by i.p. and i.m. routes resulted in comparable T cell stimulatory capacities of overlapping N-specific peptides from pools V8 and H8. In contrast, peptides from other pools showed no or only minor stimulatory activities, as exemplified for peptides in pools V4 and V6.

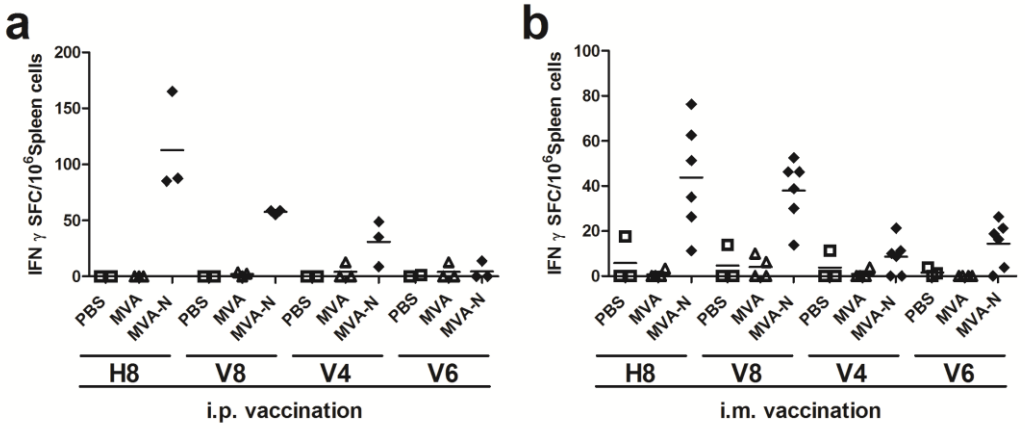

Figure 3. Screening for H2-d restricted T cell epitopes in MERS-CoV N protein using matrix peptide pools; (**a–b**) groups of BALB/c mice (n = 2 to 6) were vaccinated twice (21-day interval) by i.p. (**a**) or i.m. (**b**) application with 10^8 plaque-forming-units (PFU) of recombinant MVA-MERS-N (MVA-N). Mice inoculated with non-recombinant MVA (MVA) or phosphate-buffered saline (PBS) were used as controls. Splenocytes were restimulated in vitro with pools of overlapping peptides corresponding to MERS-CoV N protein. IFN-γ spot-forming CD8+ T cells (IFN-γ SFC) were measured by ELISPOT. The lines represent means.

Reassessment of Positive Reacting Peptides Pools for MERS-CoV N T Cell Epitopes

Following this, the peptides within the V8 and H8 peptide pools were used to elucidate in more detail the T cell epitope specificities. We subdivided the peptides from H8 and V8 in four new pools each containing five peptides (H8.1, H8.2, V8.1, V8.2). In addition, we separately tested the two 15-mer peptides #89 and #90, which were shared between pools H8 and V8. We again vaccinated BALB/c mice with MVA-MERS-N using i.p. or i.m. inoculations in a prime-boost regime. Splenocytes were prepared at day eight after the last vaccination and purified CD8+ T cells were restimulated with subpools V8.1., V8.2., H8.1, and H8.2 (Figure 4a).

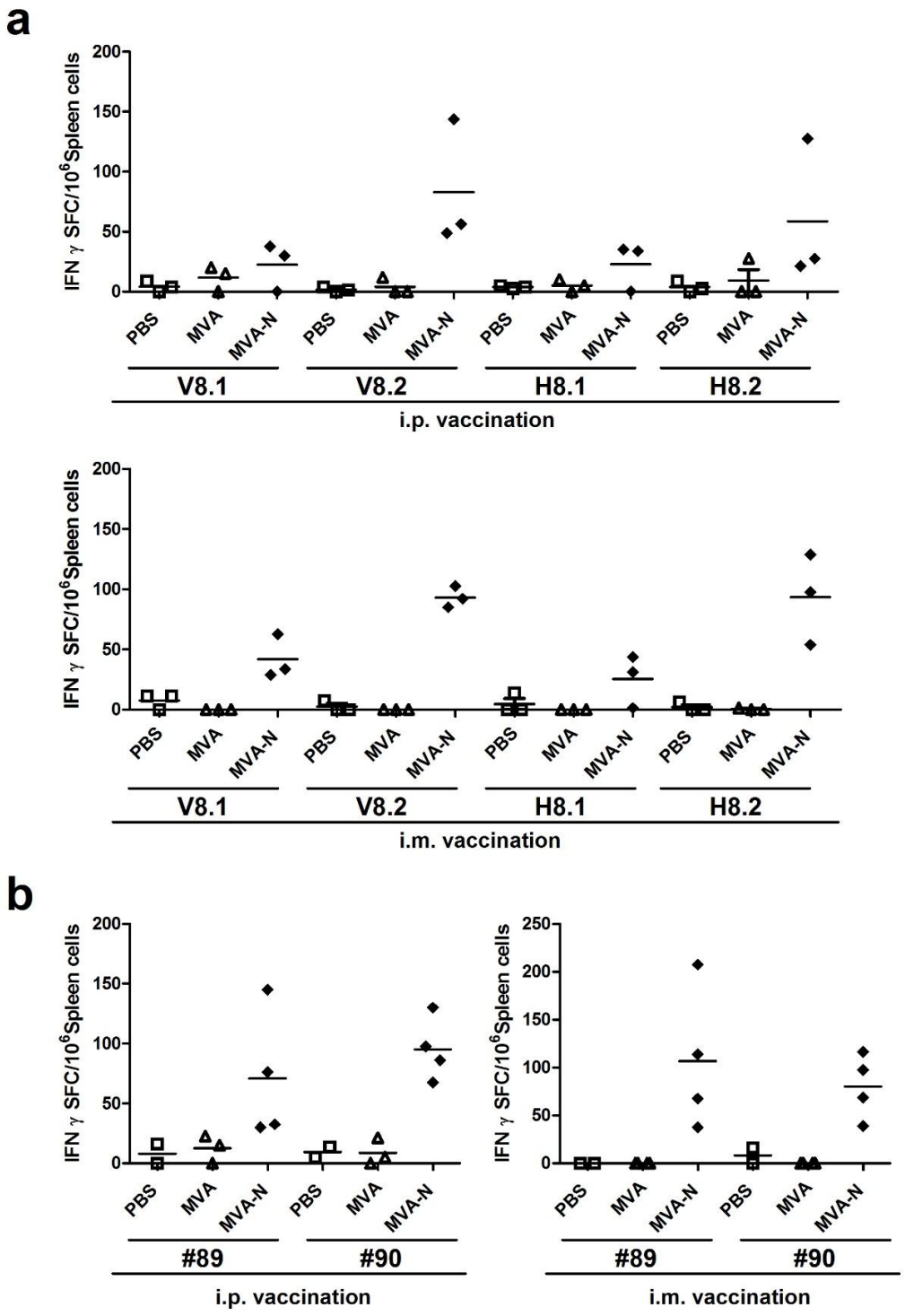

Figure 4. Mapping of H2-d restricted T cell epitopes in MERS-CoV N protein; (**a–b**) BALB/c mice (n = 2 to 4) were immunized twice (21-day interval) i.p. or i.m. with 10^8 PFU of recombinant MVA-MERS-N (MVA-N), non-recombinant MVA (MVA) or PBS. Splenocytes from vaccinated mice were incubated in the presence of subpools (V8.1, V8.2, H8.1, H8.2) from positive matrix pools (**a**) or individual 15-mers peptides #89 or #90 (**b**). IFN-γ spot-forming CD8+ T cells (IFN-γ SFC) were quantified by ELISPOT. The lines represent means.

Stimulation with peptides from pools V8.1 or H8.1 activated only minor levels of IFN-γ producing cells with mean levels about 23 SFC/10^6 splenocytes. Yet, the stimulation with subpools V8.2 and H8.2 revealed clearly higher numbers of activated T cells with 83-176 IFN-γ SFC/10^6 splenocytes. Comparable numbers of IFN-γ producing cells were again induced by i.p. or i.m. immunization. Of note, the stimulations with the 15-mer peptides #89 ($N_{353-367}$ = QNIDAYKTFPKKEKK) or #90 ($N_{357-371}$ = AYKTFPKKEKKQKAP) alone resulted in detection of substantial quantities of IFN-γ producing cells (mean levels about 71–107 SFC/10^6 splenocytes) in mice that had been vaccinated with MVA-MERS-N by both immunization routes (Figure 4b). CD8+ T cells purified from mice receiving non-recombinant MVA or mock vaccine (PBS) did not produce IFN-γ following stimulation with peptides from subpools V8.1-H8.2 and with peptides #89 and #90. When checking for the specific peptides contained within the subpools, we observed that the strongly stimulatory peptides #89 and #90 were part of the subpools V8.2 and H8.2, whereas these peptides were absent in V8.1 and H8.1. This data suggested that the overlapping 15-mer peptides #89 and #90 contained a valuable antigen epitope for the activation of MERS-CoV N-specific CD8+ T cell responses.

Identification of MERS-CoV N-specific T Cell Epitope

To map more precisely this specific epitope within the MERS-CoV N protein, we concentrated on the overlapping 11-mer peptide shared between peptides #89 and #90 and obtained nine 8-10-mer peptides (Table 1). Using these peptides for the stimulation of splenoctyes from MVA-MERS-N vaccinated mice, we obtained the highest numbers of IFN-γ producing T cells with peptide 10.2 (mean levels of 94 to 97 SFC/10^6 splenocytes), while the other peptides (10.1, 9.1, 9.2, 9.3, 8.1, 8.2, 8.3, 8.4) induced weaker responses with a mean of 5–76 IFN-γ SFC/10^6 splenocytes (Figure 5a,b).

Table 1. Peptide information.[1]

Peptide-ID	Sequence	Position
#89	QNID**AYKTFPKKEKK**	$N_{353-367}$
#90	**AYKTFPKKEKK**QKAP	$N_{357-371}$
11	AYKTFPKKEKK	$N_{357-367}$
10.1	AYKTFPKKEK	$N_{357-366}$
10.2	YKTFPKKEKK	$N_{358-367}$
9.1	AYKTFPKKE	$N_{357-365}$
9.2	YKTFPKKEK	$N_{358-366}$
9.3	KTFPKKEKK	$N_{359-367}$
8.1	AYKTFPKK	$N_{357-364}$
8.2	YKTFPKKE	$N_{358-365}$
8.3	KTFPKKEK	$N_{359-366}$
8.4	TFPKKEKK	$N_{360-367}$

[1] The common 11 amino acid sequence between positive 15-mers #89 and #90 was truncated into 8-10-mer peptides and tested by ELISPOT and ICS assay.

Furthermore, we tested the 10.2 peptide to monitor N-specific T cell responses by IFN-γ ICS and fluorescence activated cell sorting (FACS) analysis. Indeed, we could detect significant numbers of 10.2 peptide-specific CD8+ T cells being induced and activated by the MVA-MERS-N prime-boost vaccination. In comparison, the CD4+ T cell populations from splenocytes of immunized animals demonstrated only background levels of IFN-γ producing cells (Figure 5c). The vaccinia virus-specific immunodominant CD8+ T cell determinant F2L (TSCHARKE et al., 2006) served as control peptide for the detection of MVA-specific CD8+ T cells (Figure 5d).

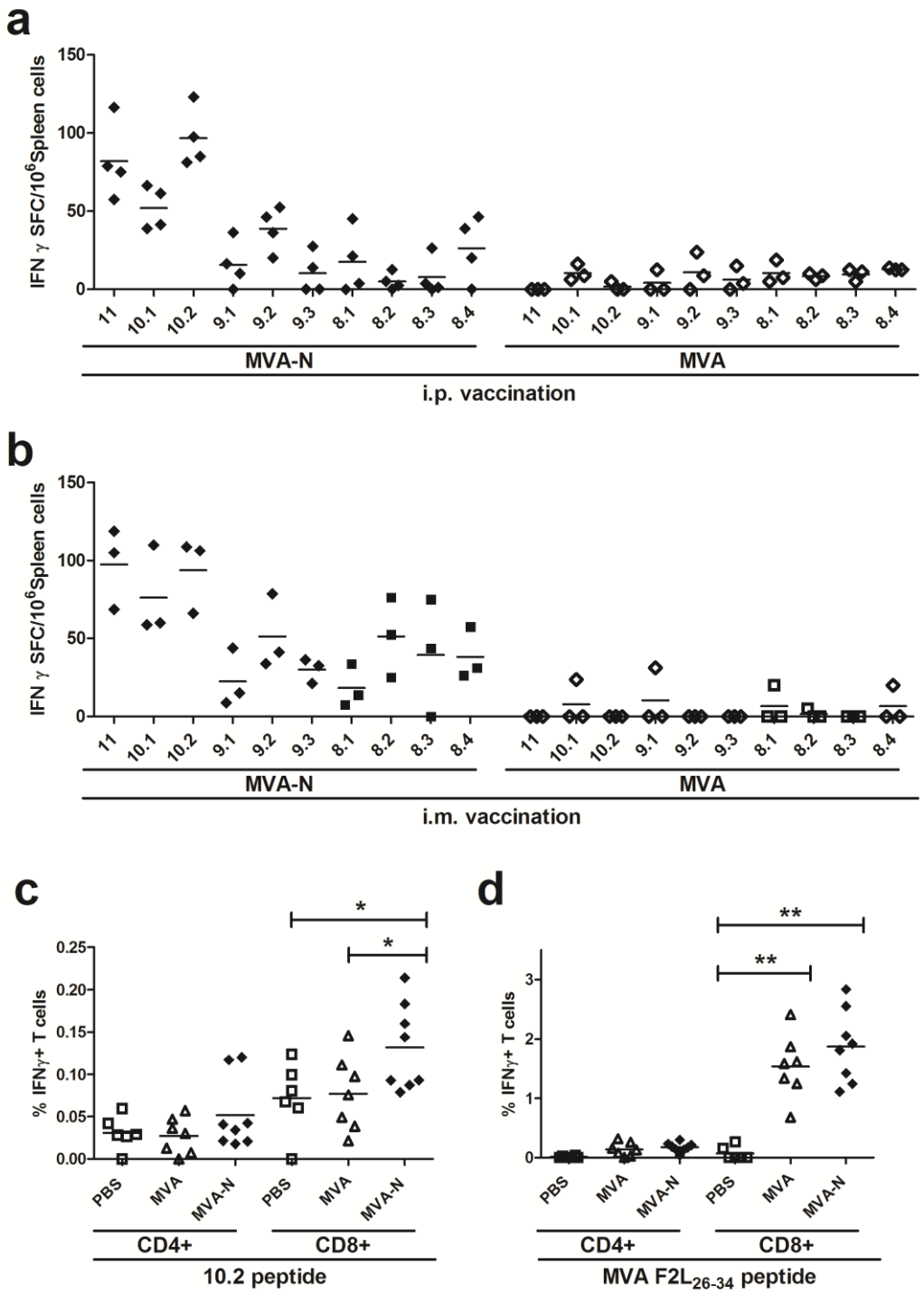

Figure 5. Identification of an H2-d restricted T cell epitope in MERS-CoV N protein; (**a–d**) Groups of BALB/c mice (n = 3 to 8) were vaccinated in a prime-boost regime with 10^8 PFU of MVA-MERS-N via i.p. (**a**) or i.m. (**b–d**) application. Mice immunized with non-recombinant MVA (MVA) and PBS served as negative controls. (**a-b**) Splenocytes were stimulated with individual 8-11-mer peptides and IFN-γ spot-forming CD8+ T cells (IFN-γ SFC) were measured by ELISPOT. (**c–d**) Splenocytes were stimulated with positive MERS-CoV N 10.2 peptide (**c**) or $F2L_{26-34}$ peptide (**d**) and IFN-γ producing CD8+ or CD4+ T cells were measured using intracellular cytokine staining assay and FACS analysis. The lines represent means. *< 0.05, **< 0.005.

Discussion

The availability of appropriate MERS-CoV-specific immune monitoring tools is a prerequisite for the successful development of vaccines and therapeutic approaches. The development of these tools is hampered by the fact that we still know little about the relevant viral antigens and the overall pathogenesis of the MERS-CoV infection. Recent studies describe a number of cases with asymptomatic MERS-CoV infection in humans and raise questions as to which factors influence the clinical manifestation of MERS (AL HAMMADI et al., 2015; SONG et al., 2018). Since asymptomatic or mild clinical manifestations of MERS-CoV infection are often associated with low levels of seropositivity, the analysis of MERS-CoV-specific cellular immune responses may facilitate further insight into the immune correlates of disease prevention. Moreover, in order to characterize the pathogenesis of MERS-CoV infection, it will be indispensable to monitor the role of virus-specific T cells in animal models of MERS and to precisely identify the antigen specificities of these T cell responses.

In the present study, we identified a major histocompatibility complex (MHC) haplotype H2-d restricted peptide epitope in the MERS-CoV N protein by stimulating T cells from MVA-MERS-N vaccinated BALB/c mice with a 2-D matrix pool of overlapping peptides. These mice have already been used in various preclinical studies to establish the MERS-CoV S protein as an important vaccine antigen for induction of virus neutralizing antibodies (SONG et al., 2013; CHI et al., 2017; COLEMAN et al., 2017; JIAMING et al., 2017; JUNG et al., 2018). Moreover, BALB/c mice transduced with the human cell surface receptor dipeptidyl peptidase 4 (hDPP4) using an adenovirus vector are susceptible to productive MERS-CoV lung infection, which allows for the testing of the protective efficacy of MERS-CoV-specific immunization using the MERS-CoV S protein (ZHAO et al., 2014; VOLZ et al., 2015b; CHI et al., 2017; COLEMAN et al., 2017; LIU et al., 2017). Here, we wished to specifically assess the suitability of an MVA-delivered MERS-CoV N antigen for the activation of cellular immune responses in mice. In general, the N protein is a well conserved internal protein and the major structural component incorporating the viral RNA within the viral nucleocapsid (NARAYANAN et al., 2000). In previous studies, the SARS-CoV N protein has also been used as a candidate antigen for vaccine development (ZHAO et al., 2005), and an experimental DNA vaccine efficiently

induced SARS-CoV N-specific cellular immunity (ZHAO et al., 2010; ZHAO et al., 2016a). In line with this data, recent studies in MERS patients demonstrated that both antibody and T cell responses are associated with recovery from MERS-CoV infection (ZHAO et al., 2017a).

The recombinant virus MVA-MERS-N produced stable amounts of MERS-CoV N antigen upon in vitro infection of human cells indicating the unimpaired expression of the target gene at the level of viral late transcription using the synthetic vaccinia virus-specific promoter PmH5 (WYATT et al., 1996). Moreover, the MERS-CoV N antigen produced in MVA-MERS-N infected cells was strongly recognized by antibodies from experimentally infected laboratory animals suggesting that N-specific immune responses were potently activated upon MERS-CoV infection. It seems noteworthy that MERS-CoV productively replicates in rabbits, but viral loads are low and the animals develop no overt disease symptoms. However, Haagmans et al. found infectious virus in the lung tissues of the rabbits and revealed the presence of the MERS-CoV N antigen in bronchiolar epithelial cells and in the epithelial cells of the nose (HAAGMANS et al., 2015). The localization of N in these respiratory epithelial cells may result in an efficient recognition by innate and also adaptive immune cells similar to those described for other viruses inducing robust protective immunity (ASCOUGH et al., 2018). This might be a possible explanation for efficient activation of MERS-CoV N-specific antibodies despite a barely productive MERS-CoV infection. Similar outcomes of infection were observed upon MERS-CoV infection in cynomolgus macaques and other relevant non-human primate models (DE WIT et al., 2013b; FALZARANO et al., 2014). Thus, the induction of N-specific immune responses in these animals emphasizes the potential usefulness of the MERS-CoV N protein to serve as a vaccine antigen.

Nevertheless, the immunogenicity of N requires further characterization in preclinical models for MERS-CoV infection. In addition to the induction of MERS-CoV-specific antibodies, the MERS-CoV N protein holds promise to efficiently activate virus-specific CD8+ T cell responses. For more detailed studies characterizing the possible role of these T cell specificities in MERS-CoV-associated immunity or pathogenesis it is highly relevant to determine the N peptide epitopes allowing for the appropriate MHC-restricted antigen presentation and the activation of virus-specific T cells. In this study, we

identified a new H2-d restricted CD8+ T cell epitope in the MERS-CoV N protein using a 2-D matrix and pools of 84 overlapping 15-mer N peptides. First, we have identified two MERS-CoV N derived peptides ($N_{353-367}$ = QNIDAYKTFPKKEKK and $N_{357-371}$ = AYKTFPKKEKKQKAP) and further mapped these 15-mer peptides to the minimal aa sequence of $N_{358-367}$ = YKTFPKKEKK representing a decamer peptide epitope (Figure S1) (PAPAGEORGIOU et al., 2016).

Figure S1. Sequence analysis and modular organization of MERS-CoV N protein. The primary amino acid sequence of MERS-CoV N protein (MERS-CoV strain EMC/2012, GenBank accession no. JX869059). The N-terminal and C-terminal domain are indicated in red and green, respectively. Predicted RNA-binding domains (aa 37-164 and aa 239-362) are highlighted in gray. The structurally flexible linker region is indicated in black containing several Ser/Arg (SR) motifs, which are underlined in black (PAPAGEORGIOU et al., 2016). H2-d restricted peptides with CD4+ ($N_{350-362}$, (ZHAO et al., 2016a)) and CD8+ ($N_{358-367}$) T cell antigenicity are underlined in orange and blue, respectively.

Analysis of MERS-CoV sequences reveals that $N_{358-367}$ is conserved among different strains of MERS-CoV (Table S2) (LEE et al., 2017).

Table S2. Comparative analysis of MERS-CoV $N_{358-367}$ epitope in different MERS-CoV strains (modified from (LEE et al., 2017)).

Lineage	Strain	$N_{358-367}$
Lineage 1	England-Qatar/2012	YKTFPKKEKK
Lineage 2	Abu Dhabi_UAE_8_2014	YKTFPKKEKK
Lineage 3	Riyadh_2014KSA_683/KSA/2014	YKTFPKKEKK
Lineage 4	Jeddah_c7770/KSA/2914-04-07	YKTFPKKEKK
Lineage 5	MERS-CoV/KOR/KNIH/0001_05_2015	YKTFPKKEKK
Outgroup	MERS-CoV EMC/2012	YKTFPKKEKK

The availability of such an epitope may allow for more detailed experimental monitoring of cellular immune responses induced by a MVA based candidate vaccine against MERS-CoV in the mouse model and potentially also in other preclinical models. Of note, the H2-d restricted CD8+ T cell epitope enables characterization of T cell responses in BALB/c mice that serve as a well-established MERS-CoV infection model following adenovirus vector mediated transduction with hDPP4 (ZHAO et al., 2014; VOLZ et al., 2015b; CHI et al., 2017; COLEMAN et al., 2017; LIU et al., 2017). A particular feature of MERS in humans, as observed upon the investigation of cluster outbreaks in hospitals, is the lack of detectable MERS-CoV neutralizing antibodies in patients with confirmed disease (WANG et al., 2015b; PALLESEN et al., 2017). This observation is attributed to the emergence of specific virus mutants evading the neutralizing antibody response, as already described for SARS-CoV (SUI et al., 2014; TAI et al., 2017). Thus, future use of a T cell-specific immune monitoring might contribute to a more detailed understanding of MERS pathogenesis. Here, studies on the function of MERS-CoV-specific CD8+ T cells in this BALB/c mouse MERS-CoV lung infection model will be helpful to better estimate the role of cellular immunity in vaccine mediated protection in MERS-CoV infection.

Finally, the MVA-MERS-N vector virus generated for this study proved to be a stable recombinant virus that can be readily amplified to obtain vaccine preparations technically fulfilling all requirements for further preclinical or even clinical development. Future work with MVA-MERS-N candidate vaccines

should help to elucidate the potential protective capacity of N-specific immune responses in MERS-CoV infections models and contribute to our better understanding of MERS vaccine-induced protection.

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V. DISCUSSION

Emerging respiratory coronaviruses such as SARS- and MERS-CoV represent potential biological threats to humans, both in terms of sporadic epidemics and possible bioterrorism. Considering the ongoing MERS-CoV epidemic and the lack of antiviral therapies, the development of safe and efficient MERS-vaccines is urgently needed. Prophylactic vaccination of people at high risk for infections, including healthcare workers or camel workers, and also the vaccination of camels, as the major animal reservoir, should be pursued to combat the ongoing spread of MERS-CoV. The MERS-CoV spike (S) protein is the major target of vaccine approaches, since neutralizing antibodies are directed against this surface protein. In accordance with data published from other coronaviruses, these S-induced neutralizing antibodies are hypothesized to correlate with protection against MERS-CoV infection (BISHT et al., 2004; SUBBARAO et al., 2004; YANG et al., 2004; CHI et al., 2017). Currently, there is rather little known about antigenic targets, which are recognized by T cells during MERS-CoV infection. However, T cells seem to be important for viral clearance and recovery from MERS disease. Based on knowledge from other coronaviruses, highly conserved (internal) structural proteins, such as the coronavirus N protein, are considered as other innovative target antigens and they may elicit a long-lived and broad-reactive T cell immune response.

MVA, a replication-deficient and safety-tested VACV, serves as a promising platform for the development of vector-based vaccines against various infectious diseases and cancer. In this study, a recombinant MVA stably expressing the full-length MERS-CoV N protein was generated to further elucidate mechanistic insights into the nature of MERS-CoV N-specific T cell immunity. T cell responses recognizing the MERS-CoV N protein were characterized in mice immunized with the MVA vaccine candidate by using overlapping peptide libraries. The identification of T cell specific epitopes may facilitate studies of immune correlates of protection and the evaluation of vaccine strategies in murine models of MERS-CoV infection.

Correlates of vaccine-induced immunity against viral infections

In general, vaccine induced protection is based on the activation of immunological memory responses, which act in combating infection or re-infection with a specific pathogen (SALLUSTO et al., 2010). Protection for most of the licensed vaccines is currently associated with the induction of strong humoral immune response as measured by neutralizing antibodies in the serum or on the mucosal surfaces (PLOTKIN, 2010). Indeed, antibodies are believed to play the key role in mediating protection against various blood-borne viruses, such as hepatitis A and B, yellow fever, measles, mumps, polio or Japanese encephalitis virus (for review: (PLOTKIN, 2008)). The induction of humoral immune response is also the main strategy of vaccines against viruses, which infect via the mucosal route, including influenza virus or rotavirus (BELSHE et al., 2000; JIANG et al., 2008; PULENDRAN & AHMED, 2011). Vaccine-induced antibodies circulating in the blood and on the mucosa build a first line of defense with the aim to control viruses in an extracellular state and to inhibit viremia (PLOTKIN, 2010). In addition to antibodies, evidence is growing that T cells also play an important role in mediating protective immunity, especially against pathogens, which are antigenically highly variable, such as influenza virus or HIV (SALLUSTO et al., 2010; PULENDRAN & AHMED, 2011). Moreover, CD4+ T cells also support the proliferation of B cells and the control of disease (PLOTKIN, 2008). The importance of pathogen-specific cellular immunity for vaccine-induced protection was confirmed in the context of influenza virus infection. Here, the efficacy of vaccination in elderly people largely depends on the induction of cellular immune response due to a decline in antibody response. Indeed, the risk of elderly people to develop severe influenza disease inversely correlates with the magnitude of influenza-specific T cell response induced by vaccination (MCELHANEY et al., 2006). Moreover, T cells are also important to control infections with human cytomegalovirus (HCMV). Here, high frequencies of virus-specific T cells correlate with the absence of HCMV complications in patients after organ transplantation (SESTER et al., 2001; BUNDE et al., 2005). Furthermore, it is known that a balanced interplay between T and B cells is important for the protection against poxviruses. For the smallpox eradication campaign, immunization with VACV induces long-term antibody response. Although antibody response declines

within the first three years after vaccination, stable amounts of VACV-specific antibodies could be detected in human vaccinees up to 75 years after immunization with VACV (EL-AD et al., 1990; HAMMARLUND et al., 2003; PUTZ et al., 2005; AMANNA et al., 2006). Such a long-lasting antibody persistence is also described in the context of Ebola virus infections. Using ELISA and plaque reduction neutralization assay, Rimoin and colleagues analyzed the blood samples from 14 patients, who had survived Ebola virus infection. Of note, they could detect neutralizing antibodies 40 years after initial infection with Ebola virus (RIMOIN et al., 2018). Back to the smallpox vaccination, beside the humoral immune response, immunization with VACV also induces a robust cellular immune response. VACV-specific T cells were found to be long-lived and stable for many decades after immunization (EL-AD et al., 1990; DEMKOWICZ et al., 1996; CROTTY et al., 2003; FREY et al., 2003). However, extensive cross sectional analysis of 306 humans vaccinated with VACV revealed that the level of antiviral CD4+ T cells is reduced slowly over time, with a half time of eight to 15 years (HAMMARLUND et al., 2003). The same is observed for the CD8+ T cell subpopulation, which declines more rapidly (AMARA et al., 2004). Taken together, these data suggest that VACV-specific antibodies as well as T cells are involved in long lasting protective immunity induced by smallpox vaccination. Thereby, protection against re-infection after vaccination is mediated by nearly lifelong persisting antibodies, which prevent severe disease. But, the presence of robust and long-term CD4+ T cells is likely to be critical for B cell proliferation and the corresponding long-lasting humoral immune response against VACV (AMARA et al., 2004; AMANNA et al., 2006). In addition, CD8+ T cells are necessary for disease control, as re-infections and disease will only be asymptomatic in the presence of both humoral and cellular immune responses (AMANNA et al., 2006; PANCHANATHAN et al., 2008; PLOTKIN, 2008). In summary, these findings indicate that vaccine-induced T cells might be an innovative and promising approach to overcome some of the more complicated viral diseases.

Advantages of MVA as vector vaccine

Several viral vectors are currently under development to be established as vaccine platform technology against infectious diseases and cancer, e.g. poxvirus, adenovirus, alphavirus or vesicular stomatitis virus. The main advantage of live viruses as a vaccine vector is their ability to infect cells and to facilitate intracellular expression of any foreign antigen (DRAPER & HEENEY, 2010). This is essential for the activation of CD8+ T cells. Since the antigenic proteins are synthesized *de novo* during the viral replication cycle within the host cell, those endogenous proteins can be processed and presented within MHC-class I molecules. Thus, viral vector vaccines efficiently induce antigen-specific CD8+ T cell responses (ALTENBURG et al., 2015). Besides cellular immune responses, the use of viral vectors allows for the activation of humoral immune responses against the antigen of choice. Thereby, it is noteworthy that the foreign antigens are expressed in their native conformation (DE VRIES & RIMMELZWAAN, 2016). In basic terms, viral vectors are distinguished into replicating and non-replicating viruses. The use of replication-competent vector viruses can be associated with severe side effects, especially in immunocompromised individuals, raising concerns about the safety of such vector-based vaccines. To address these safety concerns, attenuated or replication-deficient vector viruses have been developed, including the highly-attenuated VACV strain MVA.

The use of MVA as vector vaccine has several advantages. The capacity to insert large amounts of foreign DNA into the MVA genome enables transient expression of heterologous antigens that can induce both cellular und humoral immune responses (DE VRIES & RIMMELZWAAN, 2016). The ability of MVA to elicit high levels of antigen-specific cellular as well as humoral immune responses was demonstrated with the first recombinant MVA expressing the influenza antigens HA and NP (SUTTER & MOSS, 1992). The robust activation of cellular immune responses is supported by the fact that MVA seems to predominantly infect antigen-presenting cells, which are essential for the efficient activation of T cells. This kind of MVA cellular tropism has been demonstrated in *in vitro*, *ex vivo* and *in vivo* studies using a recombinant MVA expressing green fluorescent protein (GFP) as a reporter virus (ALTENBURG et al., 2017).

Another important characteristic of MVA-based vaccines is their high immunogenicity. Hereby it is noteworthy that immune responses and the vaccination efficacy induced by MVA vaccines are comparable to vaccines based on replication-competent VACV strains, if not even slightly better (SUTTER et al., 1994; RAMIREZ et al., 2000). This feature is most likely explained by the fact that MVA lacks several immunomodulatory proteins still expressed by the wild-type VACV (ALTENBURG et al., 2014). Due to the lack of IFN-α/β receptors, the type-I IFN response is activated during MVA infection. Indeed, Blanchard and colleagues demonstrated that MVA infection of primary human fibroblasts induces potent type-I IFN response in those cells (BLANCHARD et al., 1998). The activation of type-I IFN response by MVA was corroborated in a more recent study. Here, the infection of bone-marrow derived dendritic cells, the main producers of type-I IFNs, resulted in a strong secretion of IFN-α (WAIBLER et al., 2007). In addition to IFN-α/β receptors, MVA also lacks functional receptors for IFN-γ. This is important in the context that IFN-γ is essential for the activation of cytotoxic T cells (BLANCHARD et al., 1998). CD8+ T cells are not the only cell subpopulation, which are activated after MVA infection. Lehmann and colleagues demonstrated that intranasal infection of mice with MVA leads to immigration of monocytes, neutrophils and CD4+ T cells to the site of infection, triggered by expression of CCL2 (LEHMANN et al., 2009). Taken together these findings indicate that MVA is able to activate various components of the host innate immune system leading to immigration of several immune cells to the site of MVA-vaccine inoculation (ALTENBURG et al., 2014). The increased production of proinflammatory cytokines, such as IFN-α/β and TNF, and the immigration of different cell subpopulations provides a valuable explanation for the high immunogenicity and efficacy of MVA vaccines (BLANCHARD et al., 1998; WAIBLER et al., 2007). This intrinsic immunostimulatory capacity of MVA is comparable to adjuvants, which are used in many vaccines currently available to enhance immunogenicity and efficacy. For this reason, adjuvants are considered dispensable for the use of most MVA vaccines (ALTENBURG et al., 2014; BATISTA-DUHARTE et al., 2018).

One of the most important features of MVA-based vaccines is the excellent safety profile. This has been confirmed *in vivo* in various animal models, including immunocompromised NHPs (STITTELAAR et al., 2001). Here, it is

remarkable that no clinical and pathological signs were noticed in immunosuppressed macaques infected with high dose of MVA (STITTELAAR et al., 2001). In addition to these promising studies in NHPs, several phase I and II clinical trials have corroborated the safety record of MVA in immunocompromised individuals being at high risk for conventional smallpox vaccination. Thereby, MVA proved to be well tolerated and immunogenic in patients with atopic dermatitis and HIV infection (VON SONNENBURG et al., 2014; GREENBERG et al., 2015; OVERTON et al., 2015).

In summary, these data highlight the suitability of MVA as vector platform for the generation of vaccines against the emerging pathogen MERS-CoV. In particular, the excellent safety profile of MVA also allows for the application in immunocompromised or elderly people. Since these individuals are at high risk to develop severe MERS disease, they represent the major target population for vaccination. Moreover, the use of MVA is highly advantageous in the development of future MERS vaccines, because both parts of the adaptive immune system can be activated efficiently. As mentioned above, this is important in the context that humoral and cellular immunity are known to be essential for long-lived and cross-reactive immunity against coronaviruses.

Immunity against viral infection of the respiratory tract: antibodies vs. T cells

According to data published in 2016 by the WHO, lower respiratory infections remained the leading cause of death due to communicable diseases, accounting for 3.0 million deaths in 2016 (WORLD HEALTH ORGANIZATION (WHO), 2016). The human respiratory tract is highly susceptible to infection with various pulmonary viruses such as influenza virus, respiratory syncytial virus, parainfluenza virus, rhinovirus or coronavirus, including the highly pathogenic SARS- and MERS-CoV (LESSLER et al., 2009; SCHMIDT & VARGA, 2018). One of the most striking feature of many respiratory viral infections, including respiratory syncytial virus and rhinovirus, is their ability to often cause re-infections throughout life, which indicates that virus-specific antibody response may decline over time (SCHMIDT & VARGA, 2018). This phenomenon is also observed in influenza virus infections. However, in contrast to virus-neutralizing antibodies, the virus-specific T cells elicited during infection with influenza A

virus subtypes are long lived and more cross-reactive than neutralizing antibodies (VAN DE SANDT et al., 2014). These long-lasting influenza-specific T cells are hypothesized to provide protection against re-infection after ages and even more against infection with new influenza viruses evolving in the future. The decline of the virus-specific humoral immune response over time has also been observed in patients infected with the highly pathogenic SARS-CoV. Here, neither antibodies nor memory B cells could be detected in recovered SARS patients six years post infection. In contrast, SARS-CoV-specific memory T cells were found to persist up to 11 years post infection (TANG et al., 2011; NG et al., 2016; OKBA et al., 2017). Currently, there is relatively little known about memory responses after MERS-CoV infection. One study showed that MERS-CoV antibody responses persisted up to 34 months, albeit reduced. However, the effect of such long-lasting antibodies on MERS-CoV re-infection and clinical severity is still unclear (PAYNE et al., 2016).

In addition to providing protection against secondary infections, T cells are also essential for the control of acute primary infections with respiratory viruses (SCHMIDT & VARGA, 2018). CD8+ T cells in particular play a crucial role in the elimination of and corresponding protection against respiratory virus infections. This phenomenon was already described more than 30 years ago in studies with mice infected with influenza virus. Here, adoptive transfer of virus-specific CD8+ T cells resulted in a significant reduction of infectious virus in the lungs following challenge infections with influenza A virus (LUKACHER et al., 1984; TAYLOR & ASKONAS, 1986; BENDER et al., 1992; SLUTTER et al., 2013). Similar studies in mice were also conducted with the highly pathogenic SARS- and MERS-CoV. Zhao and coworkers demonstrated that adoptive transfer of SARS-CoV-specific T cells into both immunocompetent or immunodeficient mice enhanced survival and reduced viral lung titers following SARS-CoV challenge infection (ZHAO et al., 2010). Furthermore, they also showed that T cell deficient mice were not able to clear MERS-CoV, whereas viral clearance was possible in mice lacking B cells (ZHAO et al., 2014). In addition, virus-specific memory CD4+ and CD8+ T cells have been confirmed to mediate protection against both SARS- and MERS-CoV infection in mice (CHANNAPPANAVAR et al., 2014b; ZHAO et al., 2016b). Evidence suggests that T cells are also important for the control of acute coronavirus infection in

humans. In SARS patients, a decreased number of T cells correlated with enhanced disease severity (LI et al., 2003; CHANNAPPANAVAR et al., 2014a).

In summary, these data highlight an important role for T cells in mediating protection against respiratory virus infections, including the emerging pathogen MERS-CoV. Moreover, it is indispensable to identify the role of MERS-CoV specific T cells and their antigen-specificities for understanding MERS-CoV pathogenesis. From SARS-CoV it is known that low levels of virus-specific T cell immune response is responsible for severe disease and pathological changes in SARS-CoV infected mice (ZHAO et al., 2009; ZHAO et al., 2010). With regard on vaccine development, future improved vaccination strategies against MERS-CoV should target both arms of the adaptive immune response, virus-specific T cells as well as neutralizing antibodies, to induce long-lived protective immunity and to prevent severe MERS disease (SCHMIDT & VARGA, 2018).

Challenges for the development of vaccines against MERS-CoV and emerging coronaviruses in general

As already mentioned above, approaches to develop candidate vaccines against the highly pathogenic MERS-CoV mainly focused on the S protein, which plays an important role in viral infection and pathogenesis and represents the major target for neutralizing antibodies (MOU et al., 2013; OKBA et al., 2017). Here, the RBD within the MERS-CoV S protein is of particular interest for the development of MERS-CoV specific vaccines, including recombinant proteins, nanoparticles or virus-like particles (ZHOU et al., 2019). One of the most striking features of MERS-CoV pathogenesis in humans is the tendency of MERS-CoV RBD to mutate as a result of viral evolution (ZHANG et al., 2016; TAI et al., 2017). Several important key mutations, also called “antibody escape mutations”, have been identified in the RBD of different MERS-CoV strains from infected humans and dromedaries (TANG et al., 2014; KIM et al., 2016; TAI et al., 2017). Such antibody escape MERS-CoV mutants may facilitate viral evasion of cross-neutralizing antibodies raising concerns about the efficacy of S protein-based vaccine candidates (TAI et al., 2017). Thus, in light of ongoing outbreaks caused by MERS-CoV, it is important to develop vaccines with more broad-reactivity against coronaviruses evolving in the future. In that context, it is

also noteworthy to mention that the S protein, especially the RBD within the S1 subunit, is highly divergent among the different coronaviruses (OKBA et al., 2017). As a result, S-specific neutralizing antibodies primarily target homologous strains (CHANNAPPANAVAR et al., 2014a). Due to the limitations of coronavirus-specific antibody responses, the induction of long-lived T cell mediated immune responses should be pursued via a more broadly activating coronavirus antigen. Therefore, internal structural proteins, such as the MERS-CoV N-protein, are promising target immunogens as they are more conserved among different coronaviruses (OKBA et al., 2017). The potential of internal structural proteins to activate T cells with broad reactivity has already been shown in the context of immunity against different influenza A virus subtypes. In that context, the highly conserved internal proteins NP and M1 were shown to induce appropriate levels of cross-reactive T cell response against different subtypes of seasonal influenza A viruses (YEWDELL et al., 1985; VAN DE SANDT et al., 2014; LIU et al., 2016; ZHAO et al., 2017b). In addition, these CD8+ T cells directed against NP and M1 protein of seasonal influenza A virus showed cross-reactivity with influenza viruses of avian or swine origin (JAMESON et al., 1999; KREIJTZ et al., 2008; HILLAIRE et al., 2013; ALTENBURG et al., 2014; VAN DE SANDT et al., 2014). Currently licensed seasonal influenza (Flu) vaccines focus on the robust activation of strain-specific antibodies targeting the viral surface proteins HA and neuraminidase (NA). The most commonly used Flu vaccines are available as trivalent inactivated vaccine formulations. These trivalent inactivated vaccines contain three virus strains, including two influenza A viruses (H1N1 and H3N2) and one influenza B virus. In more recent years, due to the spread of two antigenically different influenza B viruses, quadrivalent inactivated vaccine formulations have been generated, including a second influenza B virus strain (RAJAO & PEREZ, 2018). In addition to inactivated vaccine formulations, live-attenuated and recombinant HA vaccines are available that also induce neutralizing antibodies against HA and NA (YAMAYOSHI & KAWAOKA, 2019). Indeed, both influenza surface proteins efficiently induce influenza A virus-neutralizing antibodies, which correlate with protection. However the HA and NA encoding genes are frequently mutating as a result of selective pressure exerted by previous antibody inducing vaccinations or infections (ALTENBURG et al., 2015). Thus,

such Flu vaccines that are based on HA- and NA-mediated immunity require an annual update to provide solid protection against antigenically mismatching seasonal influenza A virus strains (SMITH et al., 2004). Focusing more on the conserved internal influenza antigens may help to overcome the limitations of influenza candidate vaccines solely based on HA and NA.

The importance of long-lived immunity is further highlighted by the key target populations for MERS-CoV vaccination. Since most of the larger MERS-CoV outbreaks occurred in hospitals also involving insufficient hygiene measures, healthcare workers being at high risk for MERS-infections would benefit from robust and long-lived T-cells against MERS-CoV. Considering emergency scenarios, short-term protection with a rapid onset of protective immunity might be another important requirement for a promising candidate vaccine against MERS-CoV. Besides healthcare workers, people working with dromedary camels in endemic areas are another important target population for vaccine application (OKBA et al., 2017). Ideally, the same vaccine should be suitable for vaccination of dromedary camels to prevent primary zoonotic infections humans. So far, two MERS candidate vaccines have been evaluated in dromedary camels. Both candidate vaccines are based on the MERS-CoV S protein (MUTHUMANI et al., 2015; OKBA et al., 2017). The first one, a DNA vaccine, proved to be immunogenic in camels by inducing binding as well neutralizing antibodies (MUTHUMANI et al., 2015). The second candidate vaccine, is based on the viral vector MVA (MVA-MERS-S) (SONG et al., 2013; VOLZ et al., 2015b). Of note, immunization of camels with the MVA-MERS-S vaccine induced protective immunity against MERS-CoV challenge infection. Thereby, the protection correlated with neutralizing antibodies on mucosal surfaces (HAAGMANS et al., 2016). At present, this MVA vaccine is being tested in phase I/IIa clinical trials for the application in humans. The promising results of preclinical and clinical MVA-MERS-S testing encouraged us to utilize the same vector platform for the investigation of immune responses against the MERS-CoV N protein. In addition to the S protein, the conserved N protein may be used as a second co-expressed antigen in the MVA vector system to address the limitations of S-mediated humoral immune responses. For this purpose, we first generated a recombinant MVA that showed stable expression of MERS-CoV N protein, as demonstrated by Western Blot analysis. Moreover,

the new recombinant MVA-MERS-N vaccine proved to be replication deficient in mammalian cells, which confirmed the expected safety profile of recombinant MVA vaccines. Immunization studies in mice with MVA-MERS-N nicely show that the new recombinant MVA vaccine is able to induce a MERS-N-specific T cell response.

Significance of virus-specific T cell epitopes in the development of vaccines

The knowledge about virus-specific epitopes for T cells and B cells offers a broad application area in immunology and also microbiology. Here, the availability of specific epitopes facilitates mechanistic insight into disease specific pathogenesis. Moreover, in the context of vaccinology, identification of virus-specific epitopes allows for immune monitoring during vaccination studies in preclinical models. Furthermore, ongoing research focuses more and more on the development of epitope-based subunit vaccines, which are based solely on a single or the combination of different epitopes for the induction of stronger protective and maybe broad-reactive immunity (SANCHEZ-TRINCADO et al., 2017). Epitope-based vaccines have several advantages compared to vaccines based on full-length antigens. They are highly stable, they can be easily produced, and they are less expensive than full-length antigen vaccines (AGGARWAL et al., 2019). In the last years, several epitope-based vaccines generated against bacteria, viruses and cancer underwent preclinical and clinical testing (KAUR et al., 2009). Inter alia, epitope-based vaccines are hypothesized as promising pre-vaccine candidates for the prevention of measles virus infection in children (EL KASMI & MULLER, 2001). Hereby, synthetic peptides representing epitopes from measles virus surface proteins proved to be protective against measles virus encephalitis in mice and they could be used for the pre-vaccination of children (OBEID et al., 1995; HATHAWAY et al., 1998; EL KASMI et al., 2000). This is possible, because the peptides are not recognized by pre-existing maternal antibodies. Thus, infants receiving the peptide-based vaccine would be protected during the gap of measles virus susceptibility until vaccination with standard live-attenuated vaccine (EL KASMI & MULLER, 2001). Another epitope-based vaccine is dealing with Bacillus anthracis. Aggarwal and coworkers demonstrated the protective efficacy of a chimeric vaccine comprising different epitopes of

Bacillus anthracis antigens. Mice treated with the epitope-based vaccine were fully protected against challenging with Bacillus anthracis (AGGARWAL et al., 2019). The results of this study show that a combination of different epitopes can lead to better protectivity. This strategy may be useful for the generation of a broad-reactive coronavirus vaccine. Using MVA-MERS-N vaccination in mice, we have identified a T cell epitope within the MERS-CoV N protein. To overcome the risk of potential antibody escape mutants, the combination of beneficial B and T cell epitopes, at best within highly conserved proteins such as the coronavirus N protein, may provide the basis for the generation of a universal coronavirus vaccine having broader protection.

Future perspectives

The studies described here using the recombinant vaccine candidate MVA-MERS-N confirmed that MVA is a good vector platform for generating safe and effective vaccines against emerging infectious diseases. Future work includes further preclinical studies in mice and other animal models to show protection against MERS-CoV induced by our MVA-MERS-N vaccine candidate.

VI. SUMMARY

Characterization of recombinant Modified Vaccinia virus Ankara expressing the Middle East respiratory syndrome coronavirus nucleocapsid protein

The Middle East respiratory syndrome coronavirus (MERS-CoV) causes severe respiratory disease and death in humans. In 2012, this novel highly pathogenic coronavirus suddenly emerged in Saudi Arabia. Since then, MERS-CoV continues to cause worldwide epidemics in humans with high morbidity and mortality. Dromedary camels are suspected to be the most important animal reservoir leading to sporadic zoonotic infections in humans, followed by intrafamilial or healthcare-associated transmission. Since virus-neutralizing antibodies directed against the coronavirus spike (S) protein correlate with protective immunity against coronavirus in general, the S protein is considered as the key immunogen for vaccine development against MERS-CoV. However, based on studies with other coronaviruses, it is well-known that T cell immune response is essential for viral clearance and recovery from MERS disease. In that context, the highly conserved and internal structural MERS-CoV nucleocapsid (N) protein is proposed as another promising target antigen for vaccine development to elicit MERS-CoV-specific T cell-based immune response.

The highly-attenuated Modified Vaccinia virus Ankara (MVA) serves as promising viral platform for the development of recombinant vector vaccines against infectious diseases and cancer in preclinical research and in human clinical trials. MVA candidate vaccines are characterized by an exceptional safety-profile and a high immunogenicity. Despite its replication deficiency in mammalian cells, MVA is able to efficiently produce foreign proteins and to activate cellular and humoral immune responses against these antigens.

The objective of this study was to characterize a recombinant MVA candidate vaccine expressing the MERS-CoV N protein. The new recombinant virus proved to be genetically stable and growth kinetics confirmed the replication deficiency of the recombinant virus on human cell lines despite the insertion of the foreign gene. In contrast, the viral replication remained unimpaired in

chicken embryo fibroblasts facilitating a large scale vaccine production. The correct and stable expression of MERS-CoV N protein in infected target cells was shown by immunoblot analysis. To assess whether the recombinant N protein can activate MERS-CoV-N-specific T cell immune responses, BALB/c mice were immunized with the MVA candidate vaccine. Overlapping peptides spanning the whole MERS-CoV N protein served to identify N-specific T cell epitopes. Using this strategy, a T cell decamer peptide epitope could be identified within the MERS-CoV N polypeptide. These results support further assessment of MERS-CoV pathogenesis and N protein-specific immune responses in mice and maybe also other preclinical animal models.

VII. ZUSAMMENFASSUNG

Untersuchung von rekombinantem Modifizierten Vacciniavirus Ankara zur Expression von Nucleokapsid Protein des Middle East Respiratory Syndrome Coronavirus

Das Middle East Respiratory Syndrome Coronavirus (MERS-CoV) ist in der Lage schwere Atemwegserkrankungen mit oftmals tödlichem Verlauf im Menschen hervorzurufen. Dieses neue hochpathogene Coronavirus konnte erstmals im Jahre 2012 bei einem Patienten in Saudi-Arabien isoliert werden. Seitdem verursacht das MERS-CoV immer wieder weltweit Ausbrüche, die von hoher Erkrankungs- und Sterberate gekennzeichnet sind. Dromedare sind bisher als einziges Tierreservoir identifiziert worden und gelten als Quelle für zoonotische Infektionen beim Menschen, die wiederum zur Weiterverbreitung des Virus innerhalb von Familien und in Krankenhäusern führen können. Im Allgemeinen korrelieren neutralisierende Antikörper gegen das Coronavirus Spike (S) Protein mit schützender Immunität gegen Coronavirusinfektionen. Daher gilt das S Protein als wichtigstes Immunogen für die Entwicklung von MERS-Impfstoffen. Allerdings ist basierend auf Untersuchungen von anderen Coronaviren bekannt, dass darüber hinaus auch eine ausreichend starke T Zell Immunantwort wichtig ist für die Viruselimination und die Erholung erkrankter MERS-Patienten. In diesem Zusammenhang wird das hochkonservierte und im Virusinneren liegende Nucleokapsid (N) Strukturprotein als ein weiteres vielversprechendes Zielantigen für die Entwicklung von MERS-Impfstoffen angesehen, die dann auch MERS-CoV spezifische T Zell-vermittelte Immunantwort hervorrufen sollen.

Das Modifizierte Vacciniavirus Ankara (MVA) gilt als eine vielversprechende Plattform für die Entwicklung von Vektorimpfstoffen gegen verschiedenste Infektions- und Krebserkrankungen, sowohl in der präklinischen Forschung als auch in klinischen Studien im Menschen. MVA Vektorimpfstoffe zeichnen sich durch exzellente Sicherheit und hohe Immunogenität aus. Trotz der fehlenden Replikation in humanen Zellen werden Fremdproteine effizient durch das Vektorvirus exprimiert. Dadurch kommt es zur Aktivierung von zellulärer und auch humoraler Immunantworten auch gegen die Fremdantigene.

Ziel dieser Arbeit war es, einen neuen auf MVA basierenden Kandidatimpfstoff gegen das MERS-CoV zu entwickeln und zu charakterisieren, der das MERS-CoV N Protein exprimiert (MVA-MERS-N). Molekularbiologische Charakterisierung *in vitro* in Zellkultur bestätigte die genetische Stabilität des neu generierten MVA-MERS-N. Des Weiteren wurde mittels Wachstumsanalysen die Replikationsdefizienz des rekombinanten MVA-MERS-N auf humanen Zelllinien nachgewiesen. Im Gegensatz dazu kann sich MVA-MERS-N auf Zellen aviären Ursprungs produktiv vermehren. Dies ermöglicht eine Impfstoffproduktion in großem Maßstab. Western-Blot-Analysen bestätigten die stabile und korrekte MERS-N Proteinsynthese in infizierten Zellen. Die Induktion einer MERS-N-spezifischen T Zell Immunantwort wurde in Impfversuchen in BALB/c Mäusen untersucht. Dazu wurden für die Identifizierung von möglichen T Zellepitopen innerhalb des MERS-CoV N Proteins überlappende Peptide eingesetzt. Mit Hilfe dieser Strategie konnten MERS-CoV N-spezifische T Zellen identifiziert werden, die ein T Zellepitop mit einer Länge von 10 Aminosäuren innerhalb des MERS-CoV N Proteins spezifisch erkennen. Diese Ergebnisse bilden die Basis für weiterführende Untersuchungen im Mausmodell und möglicherweise auch in anderen präklinischen Tiermodellen hinsichtlich der Pathogenese von MERS-CoV Infektionen und den induzierten N-spezifischen Immunantworten.

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